jove

Protocol for Human Blastoids Modeling Blastocyst Development and Implantation

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Citation

Kagawa, H., Javali, A., Heidari Khoei, H., Sommer, T.M., Sestini, G., Novatchkova, M., Scholte op Reimer, Y., Rivron, N. Protocol for Human Blastoids Modeling Blastocyst Development and Implantation. *J. Vis. Exp.* (186), e63388, doi:10.3791/63388 (2022).

Date Published

August 10, 2022

DOI

10.3791/63388

URL

jove.com/video/63388

Abstract

A model of the human blastocyst formed from stem cells (blastoid) would support scientific and medical advances. However, its predictive power will depend on its ability to efficiently, timely, and faithfully recapitulate the sequences of blastocyst development (morphogenesis, specification, patterning), and to form cells reflecting the blastocyst stage. Here we show that naïve human pluripotent stem cells cultured in PXGL conditions and then triply inhibited for the Hippo, transforming growth factor- β, and extracellular signal-regulated kinase pathways efficiently undergo morphogenesis to form blastoids (>70%). Matching with developmental timing (~4 days), blastoids unroll the blastocyst sequence of specification by producing analogs of the trophoblast and epiblast, followed by the formation of analogs of the primitive endoderm and the polar trophoblasts. This results in the formation of cells transcriptionally similar to the blastocyst (>96%) and a minority of post-implantation analogs. Blastoids efficiently pattern by forming the embryonic-abembryonic axis marked by the maturation of the polar region (NR2F2+), which acquires the specific potential to directionally attach to hormonally stimulated endometrial cells, as *in utero*. Such a human blastoid is a scalable, versatile, and ethical model to study human development and implantation *in vitro*.

Introduction

A lack of experimental models has limited the understanding of early human embryogenesis. Current knowledge of human-specific aspects of embryonic development is derived from surplus *in vitro* fertilization (IVF) embryos donated for research. However, the limited availability, difficulties of experimental manipulations, and variable quality of the embryos hinder scientific investigations. On the contrary, a faithful *in vitro* model of human embryos would allow for complex experimental manipulations, thus providing an ethical opportunity to complement the research on human

embryos[1,2,3,4]. A previously developed model of mouse blastocysts combined mouse embryonic stem cells and trophoblast stem cells[5]. In this detailed protocol, a method to generate a model of the human blastocyst from naive pluripotent stem cells that is faithful to elemental blastocyst criteria is described[6].

Four criteria for human blastoids. Here, in an attempt to establish a standardized definition of human blastoids, we propose four minimal criteria. Although not exhaustive, these criteria might serve as a basis to evaluate the parameters that permit the formation of human blastoids (**Figure 1A**). (1) Blastoids should form efficiently in terms of morphology and of generation of the analogs of the three lineages namely, epiblast (Epi), trophectoderm (TE), and primitive endoderm (PrE). Inefficiency is likely to point at an inadequate initial cell state or/and culture condition (e.g., blastoid medium). (2) Blastoids should generate analogs of the three lineages according to the developmental sequence (Epi/TE first, PrE/polarTE last)[7,8] and timing (induction ~ 3 days; embryonic days 5-7)[7,9]. (3) Blastoids should form analogs of the blastocyst stage, but not of post-implantation stages (e.g., post-implantation epiblast, trophoblast, or amnion cells). (4) Finally, blastoids should be capable of recapitulating functional features of blastocyst implantation and development. Using this protocol, human blastoids form efficiently using multiple cell lines (>70%), are able to generate the blastocyst cellular analogs sequentially and within 4 days, and the analogs are transcriptionally similar to the blastocyst stage (>96% based on several analysis)[6,10,11]. Finally, blastoids robustly generate the embryonic-abembryonic axis, which allows them to interact with hormonally stimulated endometrial cells through the polar region, and robustly expand the lineages upon extended culture (time-equivalent: embryonic day 13).

The importance of the initial cell state. Human pluripotent stem cells (hPSCs) can be stabilized in different states that attempt to capture precise developmental stages. These states are sustained by culture conditions which, although still suboptimal, constrain cells in a pre-implantation- (~ embryonic days 5-7) or post-implantation-like (~ embryonic days 8-14) epiblast stage[12]. Transcriptomic analysis showed that hPSCs cultured in PD0325901, XAV939, Gö6983, and leukemia inhibitory factor (LIF; termed PXGL naive hPSCs)[13,14] are more similar to the blastocyst epiblast as compared to hPSCs cultured in fibroblast growth factor (FGF) 2 and activin[15] (termed primed hPSCs[12]) and to human extended pluripotent stem cells (hEPSCs)[16] (see analysis in references[17,18,19]). Accordingly, the transcriptome of primed hPSCs best matches with a post-implantation/ pre-gastrulation cynomolgus monkey epiblast[20]. Additional molecular criteria, like transposon expression, DNA methylation, and X chromosome state, confirmed that variations of the naive state more closely resemble the blastocyst epiblast as compared to the primed state[17,21]. Finally, lines of naive hPSCs have been successfully derived directly from blastocysts using PXGL culture conditions[22].

Human early blastocyst cells are not yet committed. Murine lineage specification occurs from the morula stage that precedes the blastocyst stage[23]. On the contrary, dissociation and re-aggregation experiments have shown that the human trophectoderm cells of early blastocysts are not yet committed[24]. Accordingly, analysis of the cells of human blastocysts by single-cell RNA sequencing (scRNAseq) has shown that the first lineage specification (trophoblast/epiblast) occurs after the formation of the blastocyst cavity[7]. This

deferred human specification correlates with observations that hPSCs are potent to form trophoblasts[25,26,27] when mouse PSCs are largely committed to the epiblast lineage. These combined observations led to the possibility that naive hPSCs reflect a blastocyst stage and retain the potential to form the three blastocyst lineages. Lately, the potency of hPSCs to specify extraembryonic analogs has been proposed to shift from trophectoderm to amnion during progression from naive to primed state[27]. Thus, naive hPSCs are more similar to the pre-implantation stage[17,18,21] and have an enhanced capacity to form trophoblasts as compared to primed hPSCs[27], hEPSCs[16], or intermediate reprogrammed states[28], which are prone to form post-implantation analogs[10] (**Figure 1B**). The initial cell state is thus crucial to forming the appropriate extraembryonic analogs. Although a thorough side-by-side analysis of converted trophectoderm analogs remains to be done, a PXGL naive state reflecting the early blastocyst appears important to form high-fidelity blastoids.

Prompting specification and morphogenesis by signaling pathways inhibition. The inhibition of the Hippo signaling pathway is a conserved mechanism driving trophoblast specification in mice, cows, and humans[9,29,30]. Also, since 2013, it is known that the inhibition of the NODAL (A83-01) and the extracellular signal-regulated kinase (ERK; PD0325901 or equivalent) and the activation of the bone morphogenetic protein (BMP) signaling pathways triggers primed hPSCs to activate the transcriptional network associated with the trophoblast lineage[25,31,32,33,34]. Moreover, recently several reports also confirmed that the inhibition of both NODAL and ERK pathway and activation of BMP facilitate the trophoblast differentiation from naive hPSCs[25,31,32,33,34]. Finally, if trophoblast specification is triggered from a naive state, cells recapitulate aspects of the developmental progression of the trophectoderm[26]. However, self-renewing lines reflecting the blastocyst trophectoderm haven't been stabilized *in vitro*. Following trophoblast specification, induction of the epidermal growth factor (EGF) and Wnt signaling pathways along with HDAC inhibition might facilitate trophoblast developmental progression[34,35] and stabilize cells into lines of human trophoblast stem cells (hTSCs) reflecting post-implantation cytotrophoblasts[18,35]. Such lines can be derived both from blastocysts and placental tissues[35].

The second extraembryonic lineage, termed PrE, is specified after trophoblasts and originates from the epiblast[7,9]. Contrary to murine PrE[36], the human counterpart is thought to be independent of FGF signaling[37,38]. Lines reflecting the extraembryonic endoderm (termed nEnd) were established from naive hPSCs by induction of signaling pathways using activin A, Wnt, and LIF[39]. Inconsistent with embryo inhibition experiments, ERK inhibition has been shown to prevent the formation of such nEND cells *in vitro*[39]. Until now, such lines have not been derived directly from blastocysts.

Lately, models of the early embryo have been formed by combining variations of the media previously developed for hTSCs[35] and nEND cells[39] thus using activators of the transforming growth factor- β (TGF-β), EGF, and Wnt signaling pathways[28,40]. These embryo models form at low efficiency (10%-20%) and form cells resembling the post- rather than pre-implantation stage[10], including analogs of the post-implantation epiblast, trophoblast, amnion, gastrula, mesodermal tissues (~ embryonic day 14), and cytotrophoblasts[10]. On the contrary, a triple inhibition of the Hippo, ERK, and TGF-β pathways efficiently guides the formation of blastoids comprising blastocyst-like cells[41]. Along with the initial cell state, we propose that triple pathways

inhibition (Hippo, ERK, TGF-β) is the second essential parameter to form high-fidelity blastoids (**Figure 1B**).

Evaluating the cell state and reflected stage using scRNAseq. The states of cells composing blastoids can be evaluated through scRNAseq analysis. Their transcriptional similarity to specific embryonic stages can be measured using blastoid cells alone and by comparison with primed hPSCs or hTSCs that reflect post-implantation stages[20,35]. Performing cluster analyses using different levels of definition reveals how subpopulations progressively merge when definition decreases, thus revealing clusters' similarities. Although optimality in the number of clusters can be measured[42], high-resolution clustering also informs on the eventual presence of small abnormal subpopulations, for example reflecting the post-implantation stages[10]. The genes differentially expressed between clusters can provide information on their analogs in the development process by assessing the expression levels of reference gene sets that define stage-specific lineages. This allows measuring the enrichment of blastoid subpopulations either through unsupervised distance maps (e.g., using top enriched genes) or by gene set enrichment analysis (GSEA)[43]. Using this blastoid protocol, only three main clusters form that transcriptionally reflect the three blastocyst lineages. One cluster includes both the initial naive hPSCs and the epiblast analog of the blastoids. Analyzing cells at different time points showed the sequential nature of lineages specification (trophoblasts start to specify within 24 h, and primitive endoderm cells within 60 h). A high-resolution clustering captured a subpopulation of cells (3.2%) expressing genes specific to gastrulation stage embryos (possibly mesoderm or amnion). Of note, the initial naive hPSCs also comprised 5% of post-implantation-like cells, as previously described[44]. In a second analysis, blastoid cells can be merged *in silico* with reference cells isolated from concepti at different stages[45,46,47] in order to infer stage equivalence. Here, cells isolated from pre-implantation concepti[45,46], *in vitro* cultured blastocysts[45], and gastrulation-stage embryos[47] were used as reference points. Using this protocol, it was quantified that the mismatched blastoid cells revealed by high-resolution clustering indeed cluster with post-implantation mesoderm and amnion. In future steps, transcriptome benchmarking should be complemented with analysis of transposon expression, DNA methylation, and of the X chromosome status that also provide landmarks of developmental stages[21].

Evaluating axis formation and other functionalities of human blastoids. A mature blastocyst is characterized by the formation of the embryonic-abembryonic axis patterning trophoblasts for implantation. Using this blastoid protocol, an axis robustly forms exemplified by a maturation of the proximal trophoblasts (e.g., NR2F2+/CDX2-) that acquire the capacity of attaching to endometrial organoid cells only when they are hormonally-stimulated[48,49]. Comparison with trophospheres that do not form the epiblast shows that these inner cells induce abutting trophoblasts to mature so as to mediate the initial attachment to the endometrium. When cultured in an extended culture medium designed for cynomolgus monkey blastocysts[50], all three lineages from the blastoid consistently expand for six additional days (time-equivalent of day 13) although their organization doesn't reflect that developmental stage.

The implication of high-efficiency and high-fidelity human blastoids. The conservation of developmental principles that were discovered in model organisms is inherently difficult to test in the human conceptus due to the restricted access and to the technical difficulties in genetically

and physically manipulating it. A high-efficiency and high-fidelity blastoid model would allow for high-throughput genetic and drug screens, which are at the basis of scientific and biomedical discoveries. In addition, the incorporation of complex genetic modifications to alter and record biological processes would complement such studies. Overall, we propose that the triple inhibition (Hippo, TGF-β, ERK) of naive PXGL hPSCs is conductive for the efficient formation of high-fidelity human blastoids complying with the four minimal criteria. The scalable and versatile nature of this protocol makes it suitable to generate targeted hypotheses that can then be validated using human blastocysts. As such, human blastoids will not replace the use of human conceptus for *in vitro* research but might act as a powerful way to funnel research through previously inaccessible experimental approaches at the heart of the scientific and biomedical discovery process. The protocol shows how to form human blastoids and also how to analyze the cells that are contained within the blastoid.

Protocol

The Guidelines for Stem Cell Research and Clinical Translation of the International Society for Stem Cell Research (ISSCR) recommends that research on human blastoids is permissible only after review and approval through a specialized scientific and ethics review process[3,4]. All the experimental procedures were conducted by following the guidelines of the human research ethics committee of the Institute of Molecular Biotechnology of the Austrian Academy of Science (IMBA) under the approval Rivron_Stellungnahme_2020-04-22. Compliance with these guidelines is necessary for publishing research results in scientific journals.

1. Culture of human naive embryonic stem cell in PXGL condition

NOTE: Naive hPSCs can be obtained from relevant laboratories. Lines used here were obtained from the laboratories of Yasuhiro Takashima (currently at CiRA, Kyoto, Japan) and of Austin Smith (currently at Living Systems Institute, Exeter, UK). Alternatively, naive hPSCs can be resetted *in house* from lines of primed hPSCs as described previously[13,14]. Naive hPSCs appear stable for multiple passages (> 15) but the quality of the culture can vary over time. If the quality of naive hPSC decreases, thaw a new vial of cells or generate *de novo* naive hPSCs from primed PSCs. For all media compositions see **Supplementary Table 1**.

1. Preparation of irradiated mouse embryonic feeder (MEFs) layer
    1. The day before passaging of naive hPSCs, prepare a 6-well cell culture plate with irradiated MEF layers by following the steps described below.
    2. Coat a 6-well cell culture plate with 1 mL of 0.1% gelatin in PBS per well. Incubate the plate at 37 °C for 30 min. Remove the gelatin solution.
    3. Prepare MEF medium at 37 °C.
    4. Thaw MEFs in a water bath at 37 °C until only a small ice clump is left. Dissolve the volume of the vial with 1 mL of prepared MEF medium using a P1000 pipette.
    5. Transfer the cell suspension into a 15 mL tube. Spin down the suspension at 200 x *g* for 4 min. Aspirate the supernatant and resuspend the MEF pellet by adding fresh MEF medium (sufficient for 1.5 mL per well).

6. Count the cells using cell counting slides and add 300,000 cells per well and transfer the plate into a normoxy incubator at 37 °C.

   NOTE: If MEFs detach over time, fresh MEFs can be added into the PXGL medium during routine media change.

2. Passaging of human naive pluripotent stem cells

   1. Before passaging hPSCs, check their morphology under the microscope. Colonies typically have a dome-shaped morphology with bright, defined borders. If the individual colonies show a flatter morphology or if differentiated colonies start to emerge, follow the instructions of step 1.2.8.

   2. Aspirate the medium and wash cells with PBS once. Add 500 µL of cell detachment solution per well of a 6-well plate.

   3. Incubate the cells for 5 min at 37 °C. Use a P1000 pipette and pipette the cells several times to dissociate the colonies into single cells.

   4. Collect the cells and transfer them into a 15 mL tube containing washing buffer (1 mL per well of a 6-well plate). Spin down the cells at 200 x *g* for 4 min.

   5. Aspirate the supernatant and resuspend the pellet in fresh PXGL medium (sufficient for 1.5 mL per well). Consider a splitting ratio of 1:3-1:6 for routinely passaging.

      NOTE: After every 3-4 passages or if the quality of cell culture decreases based on the cell morphology (e.g., the emergence of flat colonies in the population), add 10 µM Y-27632 and growth factor basement membrane extract (5 µL / well) to the medium during the first 24 h after passaging.

   6. Before replating the hPSCs, prepare the plates with fresh MEFs by aspirating the MEF medium and washing the cells once with PBS. Then, use a P1000 pipette to transfer 1.5 mL of hPSCs cell suspension per well of a 6-well plate containing the MEFs.

      NOTE: Ensure that pipetting leads to a homogeneous seeding of the cells across the well area. This will ensure the growth of colonies with homogeneous sizes and relative synchrony of the cells.

   7. Culture hPSCs under hypoxic conditions at 37 °C in a humidified environment. After 24 h, hPSCs should be attached. A high number of non-adherent (or floating) cells reflect a problem of viability or of attachment upon passaging.

   8. Change medium with 1.5 mL of PXGL medium per well daily. Passage hPSCs every 3-4 days or use them for blastoid formation experiments.

      NOTE: After thawing hPSCs, passage them for a minimum of three passages before starting a blastoid experiment.

2. Formation of Blastoids

1. Formation of naive PSC aggregates

   1. Prepare and pre-warm the PXGL media, N2B27 basal media, washing buffer, PBS, and aggregation media before starting the experiment. Exclude MEFs from the hPSCs suspension for forming blastoids by following the steps described below.

   2. For MEF exclusion, prepare a gelatin-coated plate by adding 1 mL of 0.1% gelatin into the well of a 6-well plate and incubating at 37 °C for 30-90 min.

3. To harvest the cells, aspirate the medium and wash the cells with 1 mL of PBS.
4. Add 500 µL of cell detachment solution (per well of a 6-well plate) and incubate at 37 °C for 5 min.
5. Check the cells under the microscope to follow the dissociation of colonies into single cells (a few multicellular clumps can be dissociated later by gentle pipetting).
6. Dilute the cell detachment solution with 1 mL of washing buffer. Collect the cells from the plate by gently pipetting 5 to 10 times. Transfer the cell suspension into a 15 mL tube. Spin down the cells at 200 x *g* for 4 min.
7. Aspirate the supernatant, resuspend the cells in 1.5 mL of PXGL medium (per well of a 6-well plate) and seed the cells on the gelatin-coated plates for MEF exclusion and incubate at 37 °C for 60-90 min.
8. Once the naive cells are seeded for MEF exclusion, remove the PBS from microwells and equilibrate the wells with 200 µL of basal N2B27 medium (per 1 microwell chip) and incubate for 60 min at 37 °C.
9. Collect the supernatant containing the unattached naive cells, transfer it to a 15 mL tube, and spin down the cells at 200 x *g* for 4 min.
10. Aspirate the media and resuspend the cells in 1 mL of N2B27 basal media. Count the cells using cell counting slides. Spin down the cells at 200 x *g* for 4 min.
11. Aspirate the medium and add an appropriate amount of 10 µM Y-27632 contained in N2B27 media to obtain a cell density of 30,000 cells per 50 µL.

    NOTE: Optimal initial seeding cell number can vary among the different cell lines. For example, to seed 50-60 cells/microwell, 30,000 cells (including surplus considering some cells fall outside of the microwell) are seeded in 1 well of 96 well plate which contains 430 microwells. An inappropriate starting cell number can result in the small aggregate formation without cavity or formation of cavitated structure reaching more than 250 µm.
12. Aspirate the medium from equilibrated microwell arrays and add 25 µL of N2B27 media with 10 µM Y-27632. Add 50 µL of cell suspension and incubate at 37 °C for 15 min (until cells fall into the bottom of the microwell). Then, add 125 µL of N2B27 medium supplemented with 10 µM Y-27632.

2. Blastoid development
    1. Within 24 h, aggregates of naive hPSCs can be observed (day 0) on the microwell chip. To initiate blastoid formation, prepare PALLY medium and follow the steps outlined below.
    2. Pre-warm PALLY medium at 37 °C for 30 min.

       NOTE: 1-Oleoyl Lysophosphatidic acid (LPA) and 10 µM Y-27632 need to be added just before use. The optimal concentration of LPA varies between 0.5-5 µM. This has to be titrated for individual hPSC lines used for blastoids.
    3. Aspirate the aggregation medium. Add 200 µL of pre-warmed PALLY medium to the microwells. Place the cell culture plate back into a hypoxic incubator at 37 °C. Repeat the media change on day 1.

4. On day 2, remove PALLY medium and add 200 µL of N2B27 medium supplemented with LPA and 10 µM Y-27632.

   NOTE: On day 2 the majority of aggregates continue to grow. However, some aggregates form small cavities. Continuously culture blastoids in PALLY until day 4 or in *in vitro* culture medium (IVC1) from day 2 onwards. However, following this media change enhances the formation of PrE in mature blastoids.

5. Repeat media change on day 3. Complete blastoid formation occurs by day 4.

   NOTE: Blastoids are considered to have fully developed when they have undergone complete morphogenesis based on morphometrics of day 7 human blastocysts (e.g., a diameter range from 150 -250 µm; one inner cluster surrounded by an epithelium of trophectoderm-like cells) and have formed polar trophectoderm-like cells (NR2F2+/ CDX2-) and PrE-like cells (GATA4+). This can be assessed using immunofluorescence staining or fluorescence-activated cell sorting (FACS).

3. Formation of blastoids in 96-well ultra low attachment microplates

1. Prepare naive hPSCs cell suspension for blastoid formation by following the steps described above from 2.1.1 - 2.1.11.
2. Aspirate the medium and add an appropriate amount of N2B27 media containing 10 µM Y-27632 to obtain the cell density of 70 cells per 100 µL of the medium.

   NOTE: Optimal initial seeding cell number can vary among different cell lines. For example, 70 cells/well can be the optimal cell number for most cell lines.
3. Centrifuge the plate at 200 x *g* for 2 min at room temperature to cluster cells at the bottom of the wells.
4. Incubate the plate in an incubator at 37 °C under hypoxic culture conditions. Within 24 h aggregates of naive hPSCs can be observed (day 0) on the wells.
5. Prepare 2x PALLY medium. Add 100 µL of prewarmed 2x PALLY medium to the wells.
6. Place the cell culture plate back in a hypoxic incubator at 37 °C. After 24 h, aspirate half of the media (100 µL) and replace it with 100 µL of prewarmed PALLY medium. Repeat the step till day 4. Ensure not to aspirate the aggregates.

   NOTE: On day 2, the majority of aggregates continue to grow. However, some aggregates have small liquid-filled cavities. On day 4, the majority of the cavitated structures undergo complete morphogenesis to form blastocyst-like structures.

4. Formation of trophospheres

1. For trophosphere formation, follow the blastoid formation protocol from step 2.1.1 (MEF exclusion) until step 2.1.12 (last step of the seeding protocol).
2. Once aggregates of naive hPSCs have formed after 24 h, exchange the aggregation medium with PALY (without LIF) supplemented with 3 µM SC-144 for the formation of trophospheres representing early trophectoderm and PALLY supplemented with 2 µM XMU-MP-1 for the formation of trophospheres representing mature trophectoderm.
3. Refresh the medium daily. Complete trophosphere formation occurs by day 4.

5. Analysis of the blastoid cells state and its reflected stage using scRNAseq

1. To pick up blastoids and perform dissociation, warm up the shaking incubator to 37 °C and set it to 100 rpm.
2. Collect blastoids from the initial 96-well plate and transfer them to multiple wells of a U-bottom 96-well plate using a mouth pipet equipped with a glass capillary.
   NOTE: Blastoids (> 70%) should be selected based on the morphometric criteria (size = 150-250 µm with a unique inner cluster) in order to avoid contamination with non-blastoid structures (< 30%).
3. Wash once with 200 µL of PBS using a P200 by viewing under a stereomicroscope. Transfer to a well containing 50 µL of collagenase and incubate for 30 min in the shaking incubator.
4. Transfer the blastoids to a well with 100 µL of 10x trypsin-EDTA and mix well. Incubate for 20 min in the shaking incubator.
5. Dissociate the blastoids into single cell by using P200 pipette. Transfer the cells into a 15 mL tube with FACS buffer (1% FBS in PBS).
6. To capture specific ratios of the analogs of the three lineages, stain the TE and PrE analogs with TROP2 and PDGFRa antibodies respectively.
   NOTE: The number of PrE cells in human blastocysts is less as compared to mouse blastocysts, which might reflect developmental defects of blastocysts formed through *in vitro* fertilization (IVF) or a species difference. In blastoids, the PrE analogs are less abundant than the TE and EPI analogs and represent 7.4% of the cells upon counting of GATA4+ cells by immunofluorescence imaging. Also, the dissociation process might induce biases in the proportions of the different cell types as PrE analogs to represent 1%-2% of the cells upon blastoid dissociation, PDGFRa tagging and, FACS analysis.
7. FACS-sort cells from all three lineage analogs into 384-plates containing a lysis buffer for smart-seq2 analysis. Exclude dead cells marked by DAPI staining (performed according to the manufacturer's instructions).
8. To evaluate the cell states (cell type and developmental stage), compare the transcriptomic data from blastoid with the appropriate controls.

6. Extended culture to assess blastoid developmental progression

1. Culture human blastoids on basement membrane matrix-coated plates (glass bottom).
   1. Coat the plate with basement membrane matrix.
   2. Visually inspect the blastoids to assess and record morphology.
      NOTE: Only blastoids that display the classic hollow-ball blastocyst morphology with compact ICM have the potential of growing and developing further.
   3. Add 100 µL of CMRL medium-1 per one well of the 96-well plate, and place the plate in the incubator at least 2 h before blastoids transfer for equilibration.
   4. Using a stereomicroscope, visually identify the blastoids with good morphology, transfer the selected blastoids in a well of 96-well plate containing 100 µl of CMRL medium-1 to remove the traces of blastoid media.
   5. Transfer the blastoids into the well containing pre-equilibrated CMRL media-1. Place the plate in the incubator and incubate at 37°C overnight.

NOTE: Upto 5 blastoids can be cultured in one well of 96 well plates. Having too many blastoids in a single well may lead to the formation of aggregates of multiple blastoids.

6. The next day, visually inspect the blastoids under a microscope. If the blastoids are attached, add 100 µL of pre-equilibrated CMRL media-1 supplemented with 5% basement membrane matrix. Place the plate in the incubator and incubate at 37 °C overnight.

7. The next day, monitor the blastoids under a microscope. Remove half of the media (100 µL) and replace it with 100 µL of pre-equilibrated CMRL media-2 supplemented with 5% basement membrane matrix.

8. On the following days, replace half of the media (100 µL) with pre-equilibrated CMRL media-3 supplemented with 5% basement membrane matrix. Place the plate in the incubator and incubate at 37 °C overnight. Repeat every day for up to 4-6 days of *in vitro* culture.

   NOTE: We have cultured blastoids for up to 6 days in extended culture conditions which corresponds to the time equivalent of day 13 of *in vitro* cultured human embryos.

7. Immunostaining blastoids

1. Aspirate the medium. Wash samples 3x with PBS for 5 min.

2. Add 200 µL of cold 4% paraformaldehyde (PFA) in PBS and fix samples for 30 min at room temperature. Remove PFA solution and wash samples 3x with PBS for 10 min.

   NOTE: If blastoids were cultured on microwell chips, transfer the blastoids from the chip into 96-well U-bottom plates for the following steps.

3. Permeabilize and block the blastoids in 100 µL of blocking solution per well (PBS containing 0.3% Triton-X 100 and 10% normal donkey serum) for 60 min.

   NOTE: Depending on the host species of the antibodies, adapt serum accordingly.

4. Remove the blocking solution. Add 100 µL primary antibodies diluted in fresh blocking solution and incubate samples overnight at 4°C.

   NOTE: The concentrations of primary antibodies must be determined based on the manufacturer's instructions.

5. Wash samples 3x with 0.1% Triton-X 100 in PBS (washing solution) for 10 min. Add 100 µL of secondary antibodies in blocking solution together with 20 µg/mL Hoechst nuclear stain and incubate samples for 1 h at room temperature. Protect samples from light.

   NOTE: The concentrations of secondary antibodies must be determined based on the manufacturer's instructions.

6. Wash samples 3x with washing solution for 10 min. For imaging, transfer the samples onto the glass bottom µ-slide in PBS.

   NOTE: The mounting medium should be selected based on the objective used for the imaging. For example, 80% glycerol in PBS is possible to use for mounting the samples while using oil objectives.

Representative Results

Typically, naive hPSCs cultured in PXGL (**Figure 2A**) are aggregated and cavitated structures that emerge between 48 to 72 h after PALLY induction and reach a diameter of 150-250 µm within 96 h (**Figure 2B**). Using optimal (1)

seeding cell numbers, (2) duration of pre-culture aggregation with N2B27 (0 to 24 h), (3) concentration of individual chemical components (especially LPA), and (4) duration of PALLY treatment, the induction efficiency reaches 70%-80% as defined based on morphometric parameters (overall size of 150-250 µm, single regular cavity, single inner cell cluster; **Figure 2C,D**) and the presence of the three lineages. A suboptimal initial cell state and/or induction conditions will result in less efficient or no blastoid formation. To ensure maximum efficiency and to only form pre-implantation analogs, it is crucial to use a high-quality culture of naive PXGL hPSCs. This can be assessed by measuring by FACS the percentage of cells positive for the surface markers SUSD2 (naive state) and CD24 (primed state). Additional surface markers specific to the off-target extraembryonic lineages (e.g., amnion, extraembryonic mesoderm) would be useful as well but, to the best of our knowledge, are currently not available. If the formation efficiency obtained is lower than the results reported, it is important to carefully check all the components of the blastoid medium, especially LPA that is reconstituted in PBS and that, as a GPCR ligand, can be more unstable as compared to synthetic molecules reconstituted in DMSO. In most cases, even if the yield is not maximal, the cavitated structures are still composed of the three blastocyst lineages. The emergence of three blastocyst lineages and formation of embryonic-abembryonic axis can be confirmed by the immunofluorescence staining of markers (EPI: NANOG, OCT4, TE: GATA3, Polar-TE NR2F2, Mural-TE: CDX2, PrE: GATA4; **Figure 2E,G**). Trophospheres, which are only composed of TE, help to further dissect the role of intercellular communication. Trophospheres can form at 50%-60 % efficiency within 96 h of induction (**Figure 2H,I**). Blastoid formation can be performed not only in homemade microwell arrays but also in commercially available ultra-low attachment 96 well-plates with optimization of induction conditions (see Protocol and **Figure 2J**). Blastoids also have the capacity to further develop for additional 6 days, which is time-equivalent of day 13 embryo, with *in vitro* differentiation protocol (**Figure 2K**).

In order to further characterize the cell state of blastoid cells, single-cell RNA sequencing technology must be used. UMAP is commonly applied to visualize a distribution of cell states and unsupervised clustering analysis is performed on it to evaluate the proximity of individual cell states. Different parameters in the single-cell data analysis can affect how cells are displayed in the UMAPs, thus leading to clusters with different spatial and relative positions and shapes (**Figure 3A**). However, in this analysis, cells display markedly reproducibly distinct clustering profiles regardless of the parameters used to perform the clustering and the visualization of the data, which allows distinguishing with high confidence the three blastocyst lineages. We used cells from embryos harvested at different developmental stages as a reference. The merging of these datasets shows that the majority of the trophectoderm analog from blastoid clustered with pre-implantation trophectoderm but not with post-implantation trophoblasts (**Figure 3B**). These results were also confirmed by an independent consortium[10].

When Carnegie stage 7 (CS7) gastrulating embryos are introduced in the reference map, a small population of blastoid cells (3%) clustered with the mesoderm and amnion lineages of these embryos (**Figure 3C**). When amnion-like cells are introduced in the reference map, a small population of blastoid cells (< 2%) clustered with such amnion-like cells.

Overall, only the structures comprising a single regular cavity, a single inner cell cluster, an overall size ranging from 150-250 µm, comprising transcriptomic analogs of the three blastocyst lineages, and largely devoid of other lineages

(e.g., amnion, mesoderm, extraembryonic mesoderm) are considered as human blastoids.

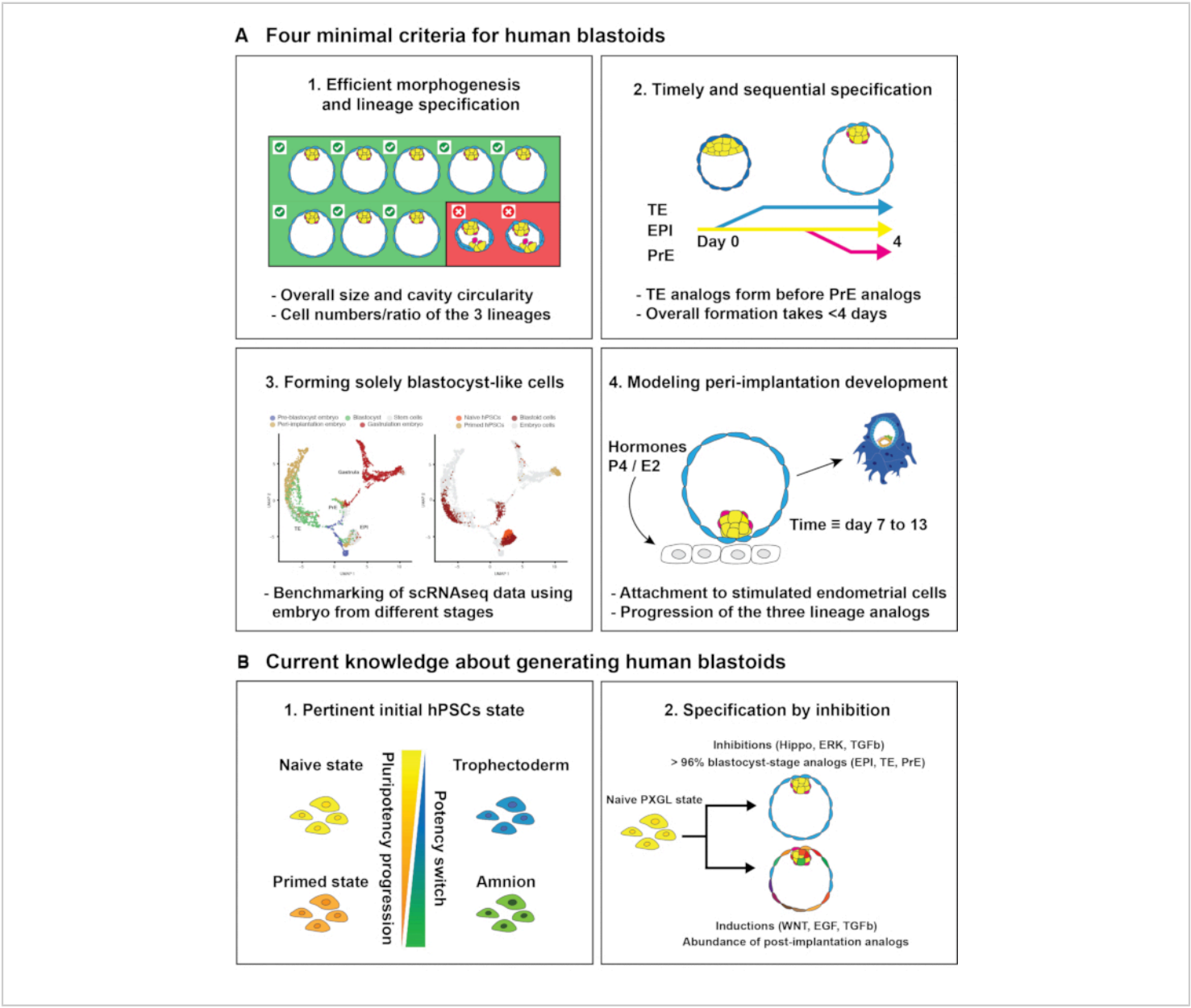

Figure 1: Four features and two approaches for generating high-fidelity blastoids. Please click here to view a larger version of this figure.

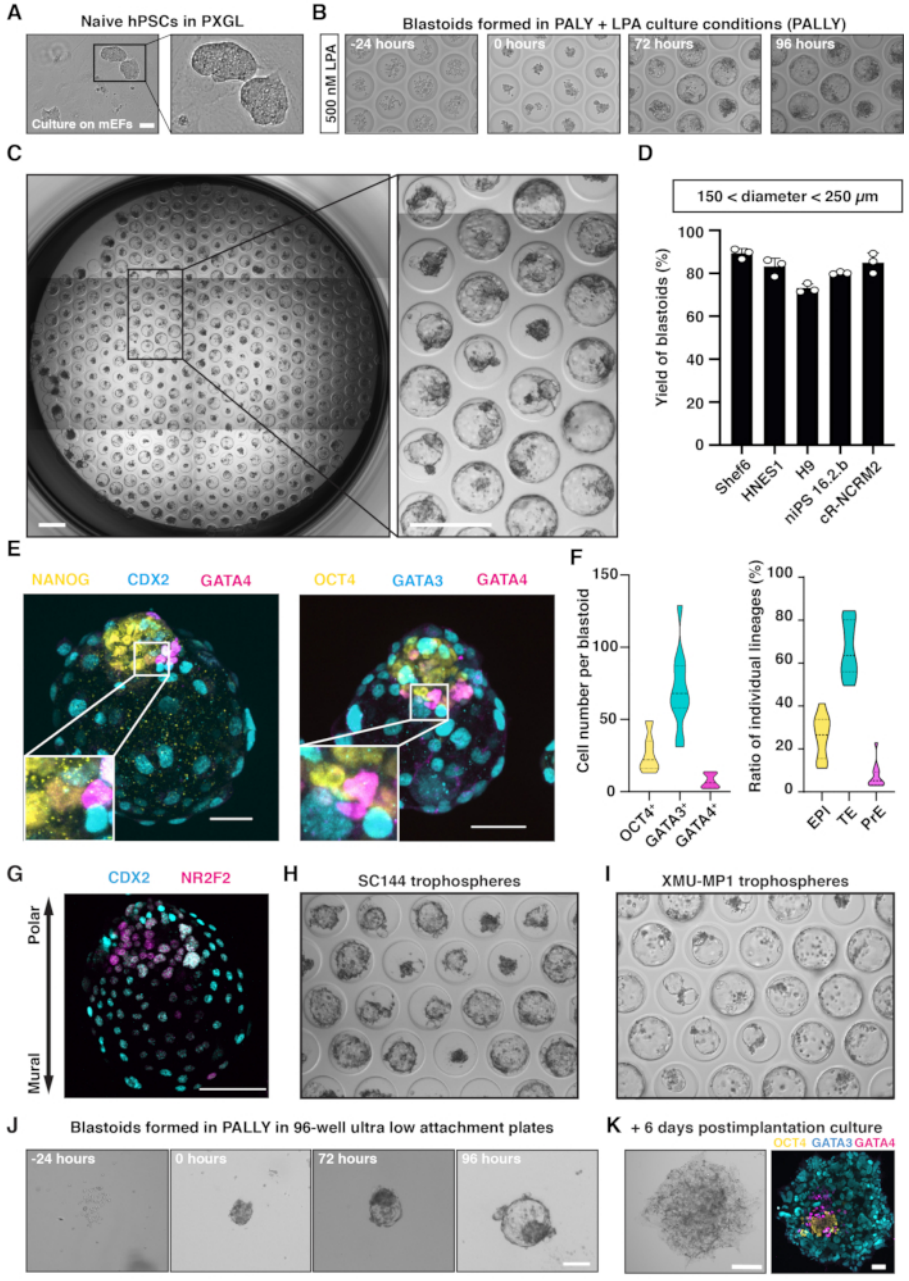

Figure 2: Blastoid and trophospheres derived from aggregates of Naïve hPSC. (**A**) Phase-contrast images showing naive hPSCs cultured in PXGL medium co-cultured with irradiated MEF. Scale bar: 50 µm. (**B**) Phase-contrast images showing the morphological change of naive hPSCs aggregates cultured on a non-adherent hydrogel microwell array with 500 nM LPA (PALLY medium). Scale bar: 200 µm. (**C**) Human blastoids formed on a microwell array after 96 h. Scale bars: 400 µm. (**D**) Quantification of the percentage of microwells containing a human blastoid induced by PALLY culture condition with optimized LPA concentration from different naive hPSC lines (n= 3 microwell arrays). (**E**) Immunofluorescence staining of human blastoids with epiblast (EPI) markers (yellow) NANOG and OCT4; the TE markers (cyan) CDX2 and GATA3; and

the primitive endoderm marker (magenta) SOX17 and GATA4. Scale bar: 100 µm. (**H-I**) Quantification of the cell number (left) and percentage of cells (right) belonging to each lineage in blastoid (96 h) based on immunofluorescence staining of OCT4, GATA3, and GATA4. (**G**) Immunofluorescence staining of human blastoids for CDX2 (cyan) and NR2F2 (magenta). (**F**) Phase-contrast images of early and late-stage trophospheres on microwell array induced by addition of 3 µM SC144 (**H**) or 2 µM XMU-MP-1 (**I**), respectively. (**J**) Phase-contrast images showing the morphological change of naive hPSCs aggregates cultured in ultra-low attachment 96 well plate with 500 nM LPA (PALLY medium). (**K**) Phase-contrast image (left) and immunofluorescence staining (right) for OCT4 (yellow), GATA3 (cyan), and GATA4 (magenta) in blastoid grown in postimplantation culture condition for 6 days. Scale bar: 100 µm. This figure is adapted from[6,10]. Please click here to view a larger version of this figure.

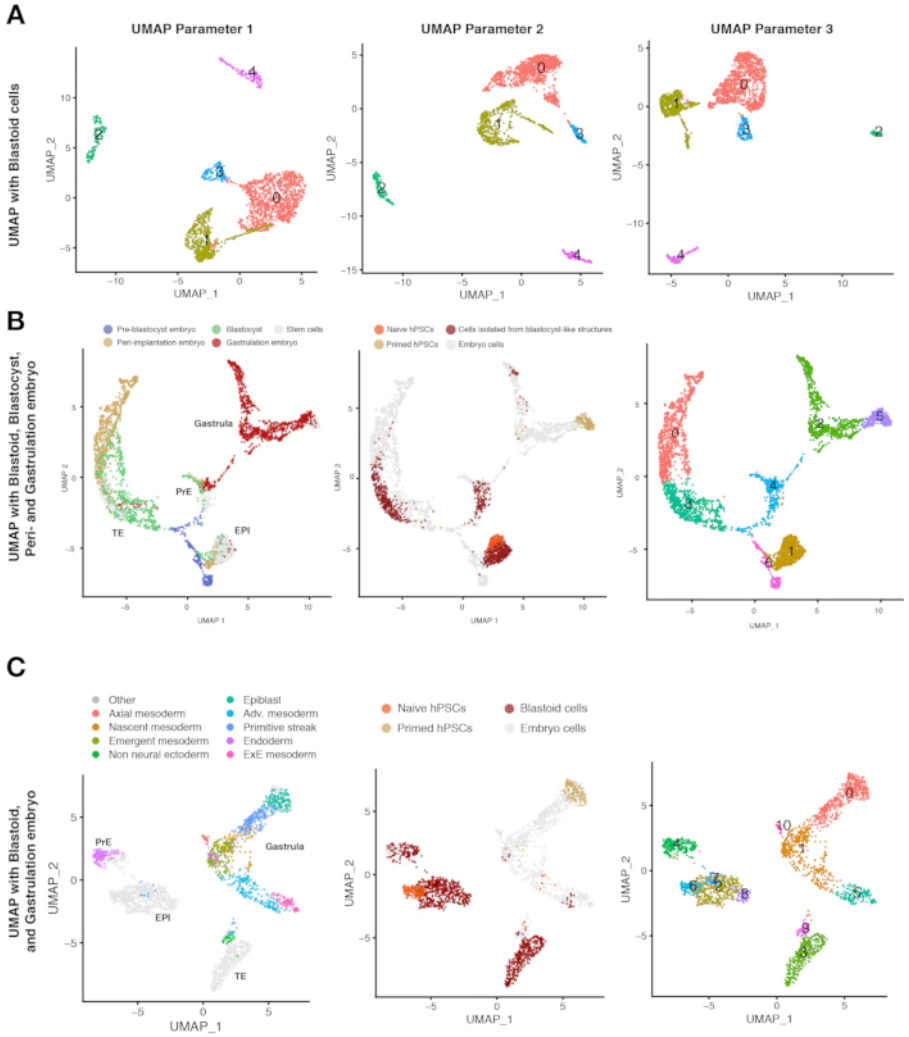

Figure 3: Characterization of the composition of blastoids by single cell sequencing. (**A**) Unsupervised clustering analysis with different parameters on UMAP of the transcriptome of single cells derived from the different time points of blastoid (24 h, 60 h, 96 h), naive hPSCs, primed hPSCs, and hTSC (represent the post-implantation cytotrophoblast). (**B**) UMAP of the transcriptome of cells derived from blastoid (96 h), naive hPSCs, and primed hPSCs integrated with published data sets from human embryos of pre-implantation, peri-implantation (*in vitro* cultured blastocysts), and gastrulation (Carnegie stage 7, i.e., between E16-19) stages. Individual cells are colored based on their origin in human embryos (left), blastoid-derived cells or stem cells (middle), and the result of unsupervised clustering analysis (right). (**C**) UMAPs of the transcriptome of cells derived from blastoids, naive hPSCs, primed hPSCs, and integrated with published data set from gastrulation (Carnegie stage 7, i.e., between E16-19) stage embryo. Individual cells are colored based on their origin in human embryos (left), blastoid-derived cells or stem cells (middle), and the result of unsupervised clustering analysis (right). This figure is adapted from[6]. Please click here to view a larger version of this figure.

Supplementary Table 1: All media composition used in this study. Please click here to download this Table.

Discussion

In the present study, we show, step by step, how to establish human blastoids with high efficiencies using a simple and robust protocol. Upon aggregation of naive PXGL hPSCs and their triple inhibition, blastoids form efficiently (> 70%) and sequentially generate the 3 blastocyst analogs within 4 days. Limitations in the efficiency and quality of the blastoids (e.g., presence of off-target cells) can occur if the initial state is sub-optimal. Of note, we have measured that PXGL hPSCs contains about 5% of cells reflecting the post-implantation stages. These cells might limit the formation of high-quality blastoids. Beyond the initial naive PXGL state that reflects the blastocyst epiblast, another pivotal factor is the medium used for blastoid formation. In order to rapidly form blastocyst-like cells and prevent the formation of off-target, post-implantation-like cells, we propose that triple pathways inhibition (Hippo, ERK, TGF-β) is essential. While different cell lines give different yields of blastoid upon ERK/TGF-β inhibition (generally around 10%-20%), exposure to LPA results in the formation of equally high blastoid yield across all cell lines, while using strict morphometric and lineage specification criteria. LPA possibly acts on the inhibition of the Hippo pathway, which plays a critical role in the first lineage segregation between epiblast and trophectoderm lineages in mouse and human[8,51]. The significant improvement of the blastoid efficiency by LPA suggests that the Hippo pathway-mediated inner-outer cell specification mechanisms at play in the blastocyst are co-opted during blastoid formation. A current limitation lies in the fact that, due to a sub-optimality of the protocols used to culture human blastocyst or blastoids at time-equivalent day 7-13 (after blastocyst/blastoid formation), we are not capable of assessing to which extent we can properly model post-implantation development.

Analyzing the transcriptomic state of the blastoid cells can easily be achieved using scRNAseq, adequate reference maps, and bioinformatic methods. Previously, the transcriptomic analysis showed that hPSCs cultured in PXGL are more similar to the blastocyst epiblast as compared to the primed state. Limitations in the analysis of the data can occur if the reference map only comprises blastocyst-stage cells. The reference map should include cells originating from post-implantation embryos in order to assess the presence of potential off-target cells. In the future, in order to benchmark blastoid cells, a reference map including all the tissues of the pre-and post-implantation human conceptus would be extremely valuable. In addition, multi-omics single cell reference maps, for example including transcriptome, chromatin accessibility, and DNA methylation, would further help. Finally, standardized bioinformatic methods to quantitatively assess the similarities between cells from embryo models and reference concepti, and to positively identify off-target cells would further help to unbiasedly analyze and compare results.

Altogether, blastoids formed by triple inhibition of Hippo, TGF-β, and ERK pathways possess the four features of 1) highly efficient morphogenesis, 2) correct sequence of lineage specification, 3) high purity of blastocyst-like cells at the transcriptome level, 4) capacity to model peri-implantation development. These features of blastoids will facilitate building hypotheses on blastocyst development and implantation, however, they do not recapitulate earlier stages of embryonic development. In contrast to the limited accessibility and versatility of human blastocyst, blastoids are amenable to genetic and drug screens for the functional

investigations of blastocyst development and implantation. In the future, such basic knowledge could contribute to improving IVF media formulation, developing post-fertilization contraceptives, and better managing early pregnancy.

Disclosures

The Institute for Molecular Biotechnology, Austrian Academy of Sciences has filed a patent application EP21151455.9 describing the protocols for human blastoid formation and the blastoid-endometrium interaction assay. HK, AJ, HHK, and NR are the inventors of this patent. All other authors declare no competing interests.

Acknowledgments

This project has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (ERC-Co grant agreement No.101002317 'BLASTOID: a discovery platform for early human embryogenesis'). H.H.K. is supported by the Austrian Science Fund (FWF), Lise Meitner Programme M3131-B. We thank Yasuhiro Takashima for sharing the H9 and H9-GFP cell lines, and Austin Smith, Peter Andrews, and Ge Guo for sharing the HNES1, Shef6, niPSC 16.2b and cR-NCRM2 cell lines. We thank Hossein Baharvand for sharing the endometrial organoids. We thank Joshua M. Brickman for sharing the RNA isolated from PrE differentiated cells and nEND cells. We thank Shankar Srinivas for sharing the single-cell RNA sequencing data of the peri-gastrulation embryo. We thank Aleksand Bykov and Luisa Cochella for technical assistance for SMARTSeq2 library preparation. We thank the NGS, Biooptic, and Stem Cell facility at IMBA for critical assistance.

References

1. Rivron, N. et al. Debate ethics of embryo models from stem cells. *Nature.* **564** (7735), 183-185 (2018).
2. Hyun, I., Munsie, M., Pera, M.F., Rivron, N.C., Rossant, J. Toward Guidelines for Research on Human Embryo Models Formed from Stem Cells. *Stem Cell Reports.* **14** (2), 169-174 (2020).
3. Clark, A.T. et al. Human embryo research, stem cell-derived embryo models and in vitro gametogenesis: Considerations leading to the revised ISSCR guidelines. *Stem Cell Reports.* **16** (6), 1416-1424 (2021).
4. Lovell-Badge, R. et al. ISSCR Guidelines for Stem Cell Research and Clinical Translation: The 2021 update. *Stem Cell Reports.* **16** (6), 1398-1408 (2021).
5. Rivron, N.C. et al. Blastocyst-like structures generated solely from stem cells. *Nature.* **557** (7703), 106-111 (2018).
6. Kagawa, H. et al. Human blastoids model blastocyst development and implantation. *Nature.* 10.1038/s41586-021-04267-8 (2021).
7. Meistermann, D. et al. Integrated pseudotime analysis of human pre-implantation embryo single-cell transcriptomes reveals the dynamics of lineage specification. *Cell Stem Cell.* 28 (9), 1625-1640.e6 (2021).
8. Gerri, C. et al. Initiation of a conserved trophectoderm program in human, cow and mouse embryos. *Nature.* **587** (7834), 443-447 (2020).
9. Gerri, C., Menchero, S., Mahadevaiah, S.K., Turner, J.M.A., Niakan, K.K. Human Embryogenesis: A Comparative Perspective. *Annual Review of Cell and Developmental Biology.* **36**, 411-440 (2020).

10. Zhao, C. et al. Reprogrammed iBlastoids contain amnion-like cells but not trophectoderm. *bioRxiv.* 2021.05.07.442980 (2021).
11. Zijlmans, D. W. et al. Integrated multi-omics reveal polycomb repressive complex 2 restricts human trophoblast induction. *Nat. Cell Biol.* **24**, 858–871 (2022).
12. Nichols, J., Smith, A. Naive and primed pluripotent states. *Cell Stem Cell.* **4** (6), 487-492 (2009).
13. Guo, G. et al. Epigenetic resetting of human pluripotency. *Development (Cambridge, England).* **144** (15), 2748-2763 (2017).
14. Takashima, Y. et al. Resetting transcription factor control circuitry toward ground-state pluripotency in human. *Cell.* **158** (6), 1254-1269 (2014).
15. Thomson, J.A. Embryonic stem cell lines derived from human blastocysts. *Science (New York, N.Y.).* **282** (5391), 1145-1147 (1998).
16. Yang, Y. et al. Derivation of Pluripotent Stem Cells with In Vivo Embryonic and Extraembryonic Potency. *Cell.* **169** (2), 243-257.e25 (2017).
17. Stirparo, G.G. et al. Integrated analysis of single-cell embryo data yields a unified transcriptome signature for the human pre-implantation epiblast. *Development (Cambridge, England).* **145** (3), dev.158501 (2018).
18. Castel, G. et al. Induction of Human Trophoblast Stem Cells from Somatic Cells and Pluripotent Stem Cells. *Cell Reports.* **33** (8), 108419 (2020).
19. Posfai, E. et al. Evaluating totipotency using criteria of increasing stringency. *Nature Cell Biology.* **23** (1), 49-60 (2021).
20. Nakamura, T. et al. A developmental coordinate of pluripotency among mice, monkeys and humans. *Nature.* **537** (7618), 57-62 (2016).
21. Theunissen, T.W. et al. Molecular Criteria for Defining the Naive Human Pluripotent State. *Cell Stem Cell.* **19** (4), 502-515 (2016).
22. Guo, G. et al. Naive Pluripotent Stem Cells Derived Directly from Isolated Cells of the Human Inner Cell Mass. *Stem Cell Reports.* **6** (4), 437-446 (2016).
23. Rossant, J. Genetic Control of Early Cell Lineages in the Mammalian Embryo. *Annual Review of Genetics.* **52**, 185-201 (2018).
24. De Paepe, C. et al. Human trophectoderm cells are not yet committed. *Human reproduction.* **28** (3), 740-749 (2013).
25. Amita, M. et al. Complete and unidirectional conversion of human embryonic stem cells to trophoblast by BMP4. *Proceedings of the National Academy of Sciences of the United States of America.* **110** (13), E1212-21 (2013).
26. Io, S. et al. Capturing human trophoblast development with naive pluripotent stem cells in vitro. *Cell Stem Cell.* **28** (6), 1023-1039.e13 (2021).
27. Guo, G. et al. Human naive epiblast cells possess unrestricted lineage potential. *Cell Stem Cell.* **28** (6), 1040-1056.e6 (2021).
28. Liu, X. et al. Modelling human blastocysts by reprogramming fibroblasts into iBlastoids. *Nature.* **591** (7851), 627-632 (2021).
29. Hirate, Y. et al. Polarity-dependent distribution of angiomotin localizes Hippo signaling in preimplantation embryos. *Current biology: CB.* **23** (13), 1181-1194 (2013).

30. Cockburn, K., Biechele, S., Garner, J., Rossant, J. The Hippo pathway member Nf2 is required for inner cell mass specification. *Current Biology: CB.* **23** (13), 1195-1201 (2013).

31. Xu, R.H. et al. BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nature Biotechnology.* **20** (12), 1261-1264 (2002).

32. Krendl, C. et al. GATA2/3-TFAP2A/C transcription factor network couples human pluripotent stem cell differentiation to trophectoderm with repression of pluripotency. *Proceedings of the National Academy of Sciences of the United States of America.* **114** (45), E9579-E9588 (2017).

33. Xu, R.H. et al. Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. *Nature Methods.* **2** (3), 185-190 (2005).

34. Horii, M., Bui, T., Touma, O., Cho, H.Y., Parast, M.M. An Improved Two-Step Protocol for Trophoblast Differentiation of Human Pluripotent Stem Cells. *Current Protocols in Stem Cell Biology.* **50** (1), e96 (2019).

35. Okae, H. et al. Derivation of Human Trophoblast Stem Cells. *Cell Stem Cell.* **22** (1), 50-63.e6 (2018).

36. Yamanaka, Y., Lanner, F., Rossant, J. FGF signal-dependent segregation of primitive endoderm and epiblast in the mouse blastocyst. *Development.* **137** (5), 715-724 (2010).

37. Kuijk, E.W. et al. The roles of FGF and MAP kinase signaling in the segregation of the epiblast and hypoblast cell lineages in bovine and human embryos. *Development (Cambridge, England).* . **139** (5), 871-882 (2012).

38. Roode, M. et al. Human hypoblast formation is not dependent on FGF signalling. *Developmental Biology.* **361** (2), 358-363 (2012).

39. Linneberg-Agerholm, M. et al. Naïve human pluripotent stem cells respond to Wnt, Nodal and LIF signalling to produce expandable naïve extra-embryonic endoderm. *Development (Cambridge, England).* **146** (24), dev.180620 (2019).

40. Yu, L. et al. Blastocyst-like structures generated from human pluripotent stem cells. *Nature.* **591** (7851), 620-626 (2021).

41. Yanagida, A. et al. Naive stem cell blastocyst model captures human embryo lineage segregation. *Cell Stem Cell.* **28** (6), 1016-1022.e4 (2021).

42. Zappia, L., Oshlack, A. Clustering trees: a visualization for evaluating clusterings at multiple resolutions. *GigaScience.* **7** (7), giy083 (2018).

43. Subramanian, A. et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America.* **102** (43), 15545-15550 (2005).

44. Messmer, T. et al. Transcriptional Heterogeneity in Naive and Primed Human Pluripotent Stem Cells at Single-Cell Resolution. *Cell Reports.* **26** (4), 815-824.e4 (2019).

45. Zhou, F. et al. Reconstituting the transcriptome and DNA methylome landscapes of human implantation. *Nature.* **572** (7771), 660-664 (2019).

46. Petropoulos, S. et al. Single-Cell RNA-Seq Reveals Lineage and X Chromosome Dynamics in Human Preimplantation Embryos. *Cell.* **167** (1), 285 (2016).

47. Tyser, R.C.V. et al. A spatially resolved single cell atlas of human gastrulation. *bioRxiv.* 2020.07.21.213512 (2020).

48. Turco, M.Y. et al. Long-term, hormone-responsive organoid cultures of human endometrium in a chemically defined medium. *Nature Cell Biology.* **19** (5), 568-577 (2017).

49. Boretto, M. et al. Development of organoids from mouse and human endometrium showing endometrial epithelium physiology and long-term expandability. *Development (Cambridge, England).* **144** (10), 1775-1786 (2017).

50. Ma, H. et al. In vitro culture of cynomolgus monkey embryos beyond early gastrulation. *Science (New York, N.Y.).* **366** (6467), eaax7890 (2019).

51. Nishioka, N. et al. The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. *Developmental Cell.* **16** (3), 398-410 (2009).