Human blastoids model blastocyst development and implantation

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Abstract

A model of human blastocyst formed from stem cells (blastoid) would support scientific and medical advances. However, its predictive power will depend on the ability to faithfully, efficiently, and timely recapitulate the sequences of blastocyst specification, morphogenesis, and patterning, and 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 (TGF- β), and extracellular signal-regulated kinase (ERK) pathways efficiently form blastoids (>70%). Within 4 days, blastoids sequentially produce analogs of the blastocyst-stage trophoblast and epiblast, followed by the formation of analogs of the primitive endoderm and the polar trophoblasts. This results in the formation of transcriptional analogs of the blastocyst (>96%) and a minority of postimplantation analogs (e.g., amnion, mesoderm). Blastoids efficiently form 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

Early mammalian embryos are notoriously difficult to study due to their scarcity and to the difficulty to manipulate them. As such, studying them remains difficult, for example, assessing the functional roles of molecules and forces in orchestrating the interplay between tissue specification, morphogenesis, and patterning.

Ethical approval

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^{2,3}. Compliance with these guidelines is necessary for publishing research results in scientific journals.

Some basic features of human blastocysts

The development of human blastocysts occurs over a period of 3 days between days 5 and 7⁴. During these days, the cells of the conceptus will form the 3 founding lineages: the pluripotent epiblast that will form the body and also some extra-embryonic tissues (e.g. amnion), and the two extra-embryonic lineages (trophoblasts and primitive endoderm) that will contribute to the formation of the placenta and yolk sac. The blastocyst consists of a trophoblast cyst englobing a unique fluid-filled cavity and a unique

inner cell cluster termed Inner Cell Mass. In the mid- to late-stage blastocyst, this ICM forms the epiblast and primitive endoderm cells. The 3 lineages thus form sequentially with the trophoblast and ICM forming first, and the epiblast and PrE forming second. A late blastocyst typically has a mean diameter of $193 \pm 18 \mu\text{m}$ ⁵.

Day 7 blastocyst (a.k.a. B5 stage)

Time of formation: 3 - 4 days

Sequence of lineage specification: Trophoblast forms first. Primitive endoderm forms last.

Cyst diameter: $193 \pm 18 \mu\text{m}$

Reagents

Media Compositions:

Medium #1: N2B27 basal media (1000ml)

Neurobasal media (in house): 469ml

DMEM/F12 (in-house): 469ml

100X N2 supplement: 5ml

50X B27 supplement: 10ml

100X Glutamax (Gibco cat# 35050038): 10ml

100mM Sodium Pyruvate (Gibco cat# 11360039): 10ml

MEM-Non-essential amino acids (Gibco cat# 11140050): 10ml

1M HEPES (in house): 10ml

50mM 2-Mercaptoethanol (ThermoFisher cat# 31350010): 2ml

100X PenStrep : 5ml

BSA*: 0 to 0.45% for naive hPSC culture and 0 to 0.3% for blastoid media

*to be optimized for individual naive hPSC cell line

Medium #2: PXGL media

PD0325901 (Medchem express cat# HY-10254): 1 μ M

XAV-939 (Medchem express cat# HY-15147): 2 μ M

Gö 6983 (Medchem express cat# HY-13689): 2 μ M

LIF (in house): 10-15ng/ml

Medium #3: PALLY media

PD0325901 (Medchem express cat# HY-10254): 1 μ M

A83-01: 1 μ M

LPA (Peprotech cat# 2256236): 0.5-5 μ M

LIF (in house): 10ng/ml

Y-27632 (Medchemexpress cat# HY-10583): 10 μ M

Medium #4: CMRL media

Basal CMRL media (100ml):

CMRL medium (Gibco cat# 21530027): 94.5ml

100X N2 supplement: 1ml

200X B27 supplement: 2ml

100X Glutamax (Gibco cat# 35050038): 1ml

100mM Sodium Pyruvate (Gibco cat# 11360039): 1ml

100X PenStrep: 0.5ml

Add 10% FBS, 20%FBS and 30% KOSR to the basal media to prepare CMRL media-1, media-2 and media-3 respectively.

Medium #5; Organoid culture media

Rspo1 conditioned medium (in-house made): 10%

Noggin-Fc-conditioned medium⁴² (in-house made): 10%

N2 supplement: 1×

B27 supplement: 1×

Insulin-Transferrin-Selenium (in-house): 1×

Glutamax: 1 μM

N-acetylcysteine (Sigma-Aldrich cat# A7250): 1.25 mM

Nicotinamide (Sigma-Aldrich cat# 72340): 2.5 mM

EGF (Peprotech cat# 100-47): 50 ng/ml

bFGF (Peprotech cat# 100-18B): 2 ng/ml

HGF (Peprotech cat# 315-23): 10 ng/ml

FGF10 (Peprotech cat# 100-26): 10 ng/ml

A83-01: 500 nM

SB202190 (Tocris cat# 1264): 10 μM

Other cell culture reagents:

Phosphate-Buffered Solution (in house)

Accutase (Biozym cat# B423201)

Matrigel® matrix growth factor reduced phenol red free (Corning cat# 356231)

Gelatin for cell culture (Sigma-Aldrich cat# G1393)

TrypleE x10 (Thermofisher cat# A1217701)

Equipment

Cell culture incubator (Normoxia: 5%CO₂ / 21%O₂/ 37°C; Hypoxia: 5% CO₂/ 5%O₂/ 37°C)

BSL-2 Biosafety cabinet

Benchtop centrifuge

EVOS M7000 Imaging System

Cell culture plasticware (plates, pipettes, cryovials, cell scrapers etc.)

Cell counting chamber

Bacteriology warmer/shaker incubator

Glass mouth pipette

Water bath

Procedure

Fabrication of microwell arrays

Microwell arrays comprising microwells were imprinted into 96-well plates as previously described⁶.

Culture of human naive embryonic stem cells in PXGL condition

Naive hPSCs can be obtained from relevant laboratories. We obtained lines from the laboratories of Yasuhiro Takashima (currently at CIRA, Tokyo, Japan) and of Austin Smith (currently at Living Systems Institute, Exeter, UK). Alternatively, naive hPSCs can be generated *in house* from lines of primed hPSCs as described previously^{7,8}. Naive hPSCs appear stable for multiple passages (>15) but the quality of the culture can vary overtime (see below). A new vial might be defrosted or *de novo* naive cells generated if the quality of the culture decreases.

Preparation of mouse embryonic feeder (MEFs) layer

The day before passaging human naive pluripotent stem cells (hPSCs), prepare a 6-well cell culture plate with MEF layers.

1.1 Coat a 6-well cell culture plate with 1ml 0.1% Gelatin in PBS per well.

1.2 Incubate the plate at 37°C for 30 min.

Prep MEF medium at 37°C.

1.3 Thaw MEFs in a water bath at 37°C until only a small ice clump is left

1.4 Dissolve the volume of the vial with 1ml of prepared MEF medium using a P1000 pipette

Transfer the cell suspension into a 15ml Falcon tube

1.5 Spin down the suspension at 400g for 4 minutes

1.6 Aspirate the supernatant and resuspend the MEF pellet by adding fresh MEF medium (count 1.5ml per well)

1.7 Remove the Gelatin solution

1.8 Add 300,000 cells per well and transfer the plate into a normoxia incubator at 37°C

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

Passaging of human naive pluripotent stem cells

Before passaging hPSCs, check their morphology under the microscope. Colonies typically have a dome shaped morphology with bright, defined borders.

2.1 Aspirate the medium and wash cells with PBS once.

2.2 Add 500ul Accutase per well of a 6-well plate.

2.3 Incubate the cells for 5 minutes at 37°C.

2.4 Use a P1000 pipette to dissociate the colonies into single cells.

2.5 Collect the cells and transfer them into a 15ml Falcon tube containing a washing buffer (1:2).

2.6 Spin down the cells at 400g for 4min.

2.7 Aspirate the supernatant and resuspend the pellet in fresh PXGL medium (count 1.5ml per well). Consider a splitting ratio of 1:3-1:4 for routinely passaging.

Note: Every 3-4 passages or if the quality of your cell culture decreased, add 10uM Y27632 and Geltrex (5ul / well) to the medium during the first 24h after passaging. Along with passaging cells when they have reached the correct colony size, this contributes to maintaining high quality hPSCs.

2.8 Before replating the hPSCs, prepare the plates of fresh MEFs by aspirating the MEF medium and washing the cells once with PBS.

2.9 Transfer the hPSCs onto the MEFs.

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

2.10 Culture hPSCs under hypoxic conditions at 37°C in a humidified environment.

Note: After 24h, hPSCs should have attached. A high number of cells in suspension reflect a problem of viability upon passaging or of attachment.

2.11 The medium should be exchanged with 1.5ml PXGL medium per well daily.

2.12 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.

Formation of Blastoids

Formation of naive PSC aggregates

3.1 Prepare and prewarm the PXGL media, N2B27 basal media, washing buffer, PBS and aggregation media before starting the experiment. MEF should be excluded from the hPSCs suspension for forming blastoids.

3.2 For MEF exclusion, prepare a gelatin coated plate by adding 1 ml 0.1% gelatin into the well of a 6 well plate and incubating at 37°C for more than 30 minutes.

3.3 To harvest the cells, aspirate the medium, wash the cells with 1ml PBS, add 500ul of Accutase (per 1 well of a 6 well plate), and incubate at 37°C for 5 minutes. Check the cells under the microscope to follow the dissociation of colonies into single cells (few multicellular clumps can be dissociated later on by gentle pipetting).

3.4 Dilute the Accutase with 1ml of washing buffer. Collect the cells from the plate by gently pipetting 5 to 10 times. Transfer the cell suspension into a 15ml tube.

3.5 Spin down the cells at 400g for 4 minutes.

3.6 Aspirate the supernatant, resuspend the cells in an appropriate amount of PXGL medium and seed the cells on the gelatin coated plates at 1:1 ratio for MEF exclusion and incubate at 37°C for 60-90mins.

3.7 Equilibration of microwells: Once the naive cells are seeded for MEF exclusion, remove the PBS from microwells and equilibrate the wells with basal N2B27 medium and incubate microwells at 37°C.

3.8 After 60 minutes of incubation, collect the supernatant containing the unattached naive cells and transfer into 15ml tube and spin down the cells at 400g for 4 minutes.

3.9 Aspirate the media and resuspend the cells in N2B27 basal media. Count the cells using countess slides.

Spin down the cells.

3.10 Aspirate the medium and add an appropriate amount of 10uM ROCK inhibitor (Y-27) contained N2B27 media. Cell concentration should be the ideal cell number per 1 well of 96 well plate in 50ul.

Note: Optimal initial seeding cell number can vary among the different cell line. As example, to seed 50-60c/uwell, 30,000 cells (including surplus considering some cells fall into outside of the microwell) are seeded in 1 well of 96 well plate which contains 430 microwells.

3.11 Aspirate the medium from equilibrated micro well and add 25 ul of N2B27 media with 10uM Y-27632.

Add 50 ul of cell suspension and incubate at 37°C for 15 minutes (until cells fall into the bottom of the microwell).

3.12 Add 125 ul of N2B27 medium supplemented with 10uM Y-27632.

Blastoid development

Within 24h, aggregates of naive hPSCs can be observed (Day 0) on the microwell chip.

3.13 Prepwarm PALLY medium at 37°C for 30min.

Aspirate the aggregation medium.

3.14 Add 200ul of prewarmed PALLY medium to the microwells

3.15 Place the cell culture plate back into a hypoxic incubator at 37°C

Repeat the media change on day 1

Note: On day 2 the majority of aggregates continue to grow. However, some aggregates develop small liquid filled cavities.

3.16 On Day 2, remove PALLY medium and exchange to N2B27 medium supplemented with LPA and Y-27632.

Note: Blastoids can be continuously cultured in PALLY until Day 4 or in IVC1 from Day 2 onwards. However, following this media change enhances formation of PrE in mature blastoids.

3.17 Repeat media change on Day 3

3.18 Blastoid formation is completed on Day 4

Note: Blastoids have undergone complete morphogenesis into blastocyst like structures, having one inner compartment surrounded by an epithelium of trophoblast cells. At this stage, blastoids can be used for downstream analysis like immunofluorescence staining and dissociation for FACS or experiments including post implantation culture.

Formation of blastoids in 96-well ultra low attachment microplates

Prepare naive hPSCs for blastoid formation by repeating Steps 3.1-3.9.

4.1 Aspirate the medium and add an appropriate amount of 10uM ROCK inhibitor (Y-27) contained N2B27 media. Cell density should be ideal for seeding optimal cell number per 1 well of 96 well in 100ul.

Note: Optimal initial seeding cell number can vary among the different cell lines. For examples 70c/well can be the optimal cell number for most cell lines

4.2 Centrifuge the plate at 200g for 2min at room temperature to cluster cells at the bottom of the wells.

4.3 Incubate the plate in an incubator under hypoxic culture conditions. Within 24h aggregates of naive hPSCs can be observed (Day 0) on the wells.

4.4 Prepare 2X PALLY medium.

4.5 Add 100ul of prewarmed 2X PALLY medium to the wells

4.6 Place the cell culture plate back into a hypoxic incubator at 37°C.

4.7 After 24h, aspirate half of the media (100 µl) and replace it with 100 µl of prewarmed PALLY medium.

CAUTION: Ensure not to aspirate the aggregates.

4.8 Repeat the previous step till day 4.

Note: On day 2 the majority of aggregates continue to grow. However, some aggregates obtain small liquid filled cavities. On day4, the majority of the cavitated structures undergo complete morphogenesis to form blastocyst-like structures.

Formation of trophospheres

Trophospheres representing either an early or more mature trophectoderm can also be formed from naive hPSCs by inhibiting the STAT pathway that is putatively important for epiblast development or by strongly inhibiting the Hippo pathway that favors trophoblast specification at the expense of epiblasts respectively. Therefore, follow the instructions of the blastoid formation protocol 3.1-3.12

5.1 Once aggregates of naive hPSCs have formed after 24h, exchange the aggregation medium with PALY (not LIF) supplemented with

a. 3 μ M SC-144 (Axon, 2324) for formation of trophospheres representing early trophectoderm

b. 2 μ M XMU-MP-1 (Med Chem Express, HY-100526) for formation of trophospheres representing mature trophectoderm

5.2 Refresh the medium daily

5.3 Trophosphere formation is completed by day 4

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

Picking up Blastoids and dissociation

6.1 Warm up the bacterial incubator/shaker to 37°C and set to 100 RPM.

6.2 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 μ m with a unique inner cluster) in order to avoid contamination with non-blastoid structures (< 30%).

6.3 Wash once with 200 μ l of PBS using a P200 and under a stereomicroscope.

6.4 Transfer to a well containing 50 μ L collagenase and incubate for 30 minutes in the shaking incubator

6.5 Transfer the blastoids to a well with 100 μ l of 10x Trypsin-EDTA and mix well

6.6 Incubate for 20 minutes in the shaking incubator

6.7 Dissociate the blastoids into single cell by using P200 pipette

6.8 Transfer the cells into the 15 ml tube with FACS buffer (1% FBS contained PBS).

6.9 To capture specific ratios of the analogs of the three lineages, the TE and PrE analogs can be stained with TROP2 and PDGFRa antibodies respectively.

Note 1: The number of PrE cells in human blastocysts is less than in mouse blastocysts, which might reflect developmental defects of blastocysts formed through IVF or a species difference. In blastoids, the PrE analogs are less abundant than the TE and EPI analogs and represent ...% of the cells upon PDGFRa immunofluorescence imaging and 1-2% of the cells upon blastoid dissociation, PDGFRa tagging and, FACS analysis.

Note 2: The dissociation process might induce biases in the proportions of the different cell types.

6.10 Cells from all three lineage analogs are FACS-sorted into 384-plates containing a lysis buffer for Smart-seq2.

Note 1: Dead cells marked by DAPI are excluded.

Note 2: To evaluate the cell states (cell type and developmental stage), transcriptomics data from blastoid should be compared with the appropriate controls. UMAP only with cells from blastoid should form three main clusters marked by the blastocyst lineage markers (Figure 3A). By merging these blastoid cells with pre- and peri- implantation blastocyst [9,10](#), a majority of TE analogs clustered with preimplantation stage TE but not with peri-implantation TE (Figure 3B). Moreover, no intermediate populations were identified in these blastoids. So-called intermediate populations are likely to reflect abnormal post-implantation cells (mesoderm or amnion). Merging with pre-gastrulation embryo data [11](#) helps to segregate blastocyst- from non-blastocyst-like cells (3.2% of total cells dissociated from blastoids)(Figure 3C).

Transfer of blastoids on the Open faced endometrial layer (OFEL) to assess their capacity to interact with endometrium

Open-faced epithelial layer (OFEL) formation from endometrial organoids

Coating the plate

7.1 Thaw Matrigel by submerging the vial in ice in a 4°C refrigerator. Place thawed vial in sterile area, spray top of vial with 70% ethanol and air dry. Using cooled pipets, mix the Matrigel to homogeneity.

7.2 Add thawed Matrigel® to the cold PBS at a ratio of 3 µL of Matrigel® to 100 µL PBS. Mix well and keep on ice. Immediately use the diluted Matrigel® solution to coat tissue culture plates.

Note: Ensure the solution evenly coats the entire bottom surface of the well; insufficient coating results in poor cell attachment.

7.3 Incubate at 37 °C for 2-3 hours.

Note: If not used immediately, seal the plate to prevent evaporation of the Matrigel® solution and store at 2 - 8°C for up to 1 week. Allow stored coated plate to warm to room temperature for 1 hour before proceeding to the next step.

7.4 Remove the Matrigel® solution using a serological pipette or by aspiration, ensuring that the coated surface is not scratched. Plates are now ready to use

Plating endometrial organoids as an OFEL

7.5 Remove the culture medium.

7.6 Add 500 µL of ice-cold DMEM-F12 to each well containing a dome to be harvested. Using a pre-coated P1000, pipette vigorously to disrupt the Matrigel® dome and resuspend the organoids.

7.7 Transfer the cells to a pre-wetted tube and centrifuge at 400g for 2 minutes at 4°C.

7.8 Discard the supernatant. Resuspend the organoids in 0.5 mL of warm (37°C) TrypLE Express dissociation medium plus ROCKi. Using a P1000, pipette up and down to mix thoroughly. Incubate the organoids at 37°C for 5 - 10 minutes.

Add 0.5 cold-DMEM+ and mix organoids.

7.9 Centrifuge the tube at 400 x g for 5 minutes.

7.10 Carefully aspirate the supernatant without disturbing the pellet and add 0.5 ml cold-DMEM.

7.11 Mix organoids thoroughly by vigorous pipetting to disrupt the organoids as much as possible. Pipetting up and down 80-100X with a P200 pipettor.

7.12 Check the organoids using a microscope to ensure sufficient disruption. Organoids should be dissociated into either individual cells or very small fragments. NOTE: If many large fragments or whole organoids remain, centrifuge at 400g for 5 minutes. Discard the supernatant. Add 0.5 mL ice-cold DMEM/F-12. Repeat pipetting until fragments are sufficiently disrupted.

7.13 Pass the digest through a 40 µm nylon mesh cell strainer (pluriSelect 43-50040-51) to purify a single-cell suspension.

7.14 Centrifuge at 400g for 5 minutes at 4°C.

7.15 Add the appropriate amount (200-300 µl) of organoid medium+ROCKi. (Add hormones to the media of stimulated organoids)

7.16 Count number of the cells.

7.17 Add 3 to 3.5 x 10⁴ cells per well into a 96-well glass bottom plate (Cellvis, P96-1.5H-N).

7.18 When EECs attached to the plate change the media with different hormonal stimulation factors and culture the cells for 2-3 day.

Blastoids transfer onto OFELs.

8.1 Remove the media and wash the wells carefully with warmed DMEM-F12. Do this very gently, otherwise the cells detached from the surface.

8.2 Add 100 µl mIVC1 media or CMRL1 media 2 hours before blastoids transfer.

8.3 Visually inspect the blastoids to assess and record morphology

Important: Only blastoids that display the classic “hollow-ball” blastocyst morphology with compact ICM have implantation potential.

Note: Only transfer blastoids onto fully confluent OFELs.

Note: Keep the plates on a heating stage in the whole procedure.

8.4 Using a stereomicroscope, visually identify the blastoids with good morphology and transfer them into the well. Place the plate in the incubator and incubate overnight.

8.5 The next day, visually inspect the blastoids under a microscope. If the blastoids are attached, remove half of the media and replace it with 100 µl of pre-equilibrated Media.

Note: For attachment efficiency calculation, remove media, wash the wells and fixed using 4% formaldehyde for 30 minutes at room temperature and subsequently process for immunofluorescence staining. The percentage of attached structures are reported as the percentage of total transferred structures.

Culture of blastoids assesses the capacity of their cells to progress

Culture of human blastoids on Matrigel-coated plates (Glass bottom)

9.1 Visually inspect the blastoids to assess and record morphology

9.2 Coat the plate with Matrigel.

9.3 Replace the media in each well with pre-equilibrated CMRL media-1.

CAUTION: Ensure not to aspirate the human blastoids.

9.4 Add pre-equilibrated CMRL media-1 to the wells and incubate for at least 2 hours before blastoids transfer.

9.5 Using a stereomicroscope, visually identify the blastoids with good morphology and transfer them into the well containing CMRL media-1. Place the plate in the incubator and incubate overnight.

9.6 The next day, visually inspect the blastoids under a microscope. If the blastoids are attached, remove half of the media (100 µl) and replace it with 100 µl of pre-equilibrated CMRL Media-1 supplemented with 5% Matrigel. Place the plate in the incubator and incubate overnight

9.7 The next day, visually inspect 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% Matrigel.

9.8 The next day, visually inspect the blastoids under a microscope. Remove half of the media (100 µl) and replace it with 100 µl of pre-equilibrated CMRL media-3 supplemented with 5% Matrigel. Repeat every

day until the desired analysis time-point is reached.

Fixing and Immunostaining of blastoids

10.1 Aspirate the medium.

10.2 Wash samples 3x with PBS for 5 min.

10.3 Add 200ul 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 U-bottom plates for the following steps

10.4 Blastoids are permeabilised and blocked in 0.3% Triton-X 100 and 10% normal donkey serum in PBS (blocking solution) for 60 minutes.

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

10.5 Add primary antibodies in blocking solution and incubate samples overnight at 4°C

Wash samples 3x with 0.1% Triton-X 100 in PBS (washing solution) for 10 min.

10.6 Add secondary antibodies (1:300) in blocking solution together with Hoechst nuclear stain (1:300) and incubate samples for 1h at room temperature.

Note: Protect samples from light

10.7 Wash samples 3x with washing solution for 10 min.

10.8 For imaging, transfer the samples onto the ibidi angiogenesis 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 could be used for mounting the samples while using oil objectives.

Troubleshooting

Time Taken

Generation of human blastoids: 3-4 days

Formation of OFELs: 10 days

Anticipated Results

A blastoid is defined based on morphological parameters of B6 blastocysts, as a cystic structure with a diameter between 150 to 250 micrometers, including a single regular cavity lined by a single layer of TE and including an inner cell cluster.

At 96 hours, >70% of the microwells contain a blastoid.

The initial cell state (naive PXGL) and the medium used to stimulate them to form blastoids (PALLY) are the two main parameters defining the successful and efficient formation of blastoids comprising analogs of the blastocyst stage and devoid of analogs of the post-implantation stages.

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Conflict of interest:

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

Ethical approvals:

Human endometrium samples were obtained from patients who signed an informed consent form and protocols approved by the Ethics Committee of Royan Institute (IR.ACECR.ROYAN.REC. 1397.93) and of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC. 1396.25). The Wicell line H9 was used under the agreement 20-WO-341 for a research program entitled 'Modeling early human development: Establishing a stem cell based 3D in vitro model of human blastocyst (blastoids)'. Blastoid generation was approved by the Commission for Science Ethics of the Austrian Academy of Sciences. This work did not exceed a developmental stage normally associated with 14 consecutive days in culture after fertilization even though this is not forbidden by the ISSCR Guidelines as far as embryo models are concerned. All experiments complied with all relevant guidelines and regulations, including the 2021 ISSCR guidelines that forbid the transfer of human blastoids into any uterus.