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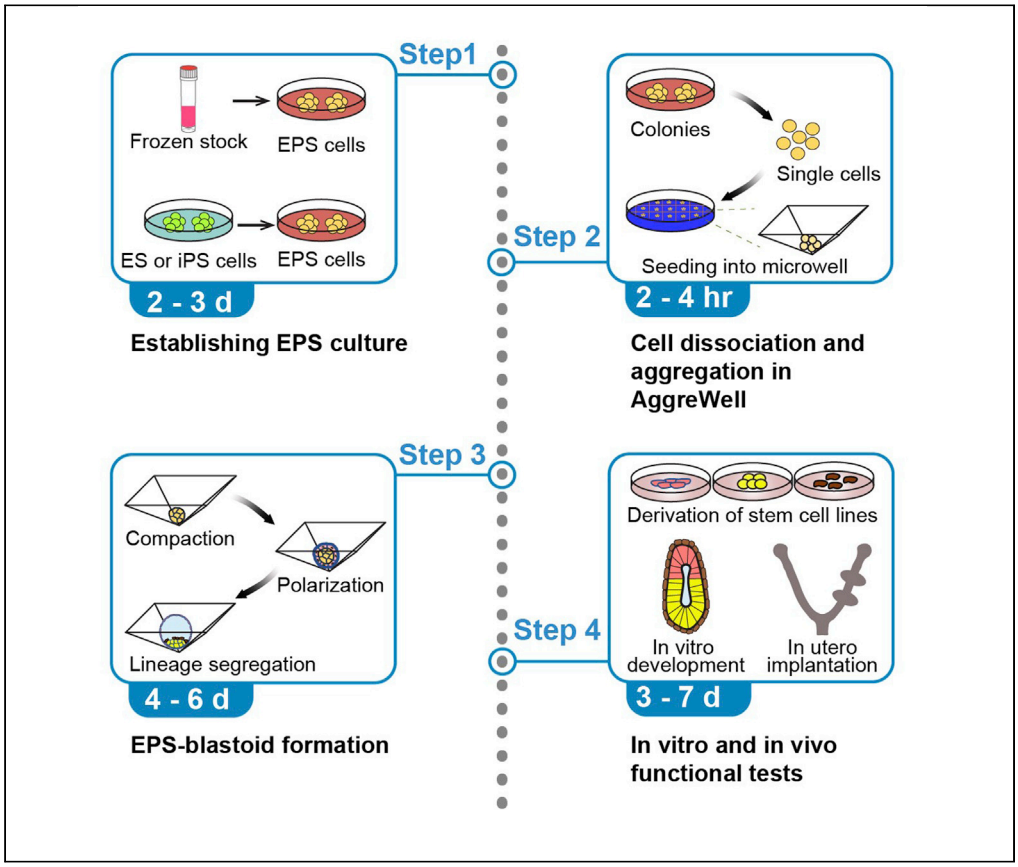
Protocol for the generation of blastocyst-like structures from mouse extended pluripotent stem cells



Extended/expanded pluripotent stem (EPS) cells can efficiently contribute to both embryonic and extraembryonic lineages *in vitro* and *in vivo*. Starting from these cells, we established a 3D differentiation system that enabled the generation of blastocyst-like structures (EPS-blastoids) through lineage segregation and self-organization. We also provide proof of concept that EPS-blastoids can be generated from adult cells via cellular reprogramming. EPS-blastoids provide a unique platform for studying early embryogenesis.

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Highlights

A protocol that enables the generation of blastocyst-like structures from EPS cells

EPS cell aggregates recapitulate early developmental events *in vitro* to form blastoids

EPS-blastoids resemble blastocysts in morphology and cell lineage allocation

EPS-blastoids are able to implant *in utero*

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Protocol for the generation of blastocyst-like structures from mouse extended pluripotent stem cells

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SUMMARY

Extended/expanded pluripotent stem (EPS) cells can efficiently contribute to both embryonic and extraembryonic lineages *in vitro* and *in vivo*. Starting from these cells, we established a 3D differentiation system that enabled the generation of blastocyst-like structures (EPS-blastoids) through lineage segregation and self-organization. We also provide proof of concept that EPS-blastoids can be generated from adult cells via cellular reprogramming. EPS-blastoids provide a unique platform for studying early embryogenesis.

For complete details on the use and execution of this protocol, please refer to Li et al. (2019).

BEFORE YOU BEGIN

The protocol below applies to EPS cells cultured in either Dr. Hongkui Deng's or Dr. Pentao Liu's protocols (Yang et al., 2017a, 2019, 2017b). See their papers for details.

The protocol below applies to embryo-derived EPS cells and ES/iPS-converted EPS cells.

Thawing and culturing EPS cells

Timing: 2–3 days

1. Gelatin coating of dishes:
   a. Make 0.1% gelatin solution in distilled H_2O and autoclave. Keep solution at room temp (20°C–25°C).
   b. Cover the bottom of tissue culture dishes with solution (1 mL for 6-well plate) and incubate at 37°C for at least 1 h. Remove gelatin solution from the plate before seeding cells.
2. Preparing MEF plate
   a. CF1 mouse embryonic fibroblasts (Passage 0) were obtained from Sciencell Research Laboratories (Catalog No.M7570-5). Grow CF1 in MEF medium (DMEM + 10% FBS + 1% NEAA + 1% Glutamax) until Passage 4.
   b. Detach MEF from the plate with TrypLE (Thermo Fisher Scientific, 1 mL/10 cm^2 area) and freeze cells at 4 million cells per vial. Treat the frozen vials with gamma X-ray irradiation (alternatively, treat cells with 10 μg/mL mitomycin C (Sigma) before dissociation and freezing). Store treated cells in liquid nitrogen.
   c. Thaw MEF vials and culture them in 0.1% gelatin-coated plate in MEF medium at about 3.3 × $10^4/cm^2$.
   d. MEF plates are ready to use 24–48 h after seeding.

3. Thawing EPS cells
   a. Take out a frozen EPS cell stock from liquid nitrogen.
   b. Quickly thaw it in a 37°C water bath until only a small piece of ice remains.
   c. Transfer the cells into a 15 mL tube containing an EPS culture medium of at least 10× the volume of the cell solution.
   d. Centrifuge at 300 *g* for 3 min.
   e. Discard supernatant and resuspend cells with 1 mL EPS culture medium ($N2B27^{LCDM}$ or EPSCM, recipes provided in "materials and equipment").
   f. Seed the cells onto MEF coated plate (e.g., 12-well plate).
4. Maintaining EPS cells
   a. Feed cells with fresh medium daily.
   b. Passage cells every 2–3 days when the colonies are big enough but not yet merging with each other.
   c. Detach EPS cells using TrypLE or AccuMAX (1 mL/10 cm^2 area) and incubate at 37°C for 3–5 min.
   d. Pipette several times to fully dissociate the cells.
   e. Centrifuge at 300 *g* for 5 min and resuspend the cells with 0.5 mL EPS culture medium.
   f. Plate 1/10 - 1/20 of the cell resuspension onto a new MEF well in EPS culture medium.

Note: EPS cell culture should be free of any type of contamination. We did not supplement antibiotics in the medium so that early signs of bacteria or fungi contamination can be spotted. We also run a PCR-based mycoplasma test biweekly. Any contaminated culture shall be discarded, and a new culture can be established from the frozen stocks. In addition, the cells should also be proliferating rapidly and be ready for split every 2–3 days at a ratio of 1: 10–1: 20. When possible, the pluripotency of EPS cells should also be tested using well-established teratoma formation or chimera assays.

Prepare the AggreWell plate

Timing: 0.5 h

5. Plate preparation.
   a. If using the plate for the first time, add a sufficient amount of Anti-adherence solution to cover the well, spin down at 2000 *g* for 1–2 min, check under a microscope to ensure bubbles are not present in the microwell, remove solution and rinse with PBS.
   b. The plate can be reused several times. Before reusing them, add TrypLE (1 mL/10 cm^2 area) and incubate at 37°C for 5 min, wash with DPBS, then add anti-adherence solution and incubate at RT for 5 min, then remove solution and rinse with DPBS.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Recombinant Human LIF	PeproTech	Cat# 300-05
CHIR99021	Reagents Direct	Cat# 27-H76
PD0325901	Selleck Chemicals	Cat# S1036
Y27632	Reagents Direct	Cat# 53-B80-50
(S)-(+)-Dimethindene maleate (DiM)	Tocris	Cat# 1425
minocycline hydrochloride (MiH)	Santa Cruz Biotechnology	Cat# sc-203339

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
SB 203580 hydrochloride	Tocris	Cat# 1402/10
Heparin sodium	Sigma-Aldrich	Cat# H3149
Verteporfin	R&D Systems	Cat# 5305/10
A83-01	Axon Medchem	Cat# 1421
BMP4	Proteintech	Cat# HZ-1040
Recombinant Human FGF4	R&D Systems	Cat# 235F4025
IWR-1-endo	STEMCELL Technologies	Cat# 72562
Recombinant Human bFGF	PeproTech	Cat# 100-18B
Puromycin	InvivoGen	Cat# ant-pr-1
Anti-Adherence Rinsing Solution	STEMCELL Technologies	Cat# 07010
IVC-1 medium	Cell Guidance Systems	Cat# M11-25
IVC-2 medium	Cell Guidance Systems	Cat# M12-25
KSOM Embryo Culture (1×)	Millipore	Cat# MR-020P-5D
N2	Thermo Fisher Scientific	Cat# 17502-048
B27	Thermo Fisher Scientific	Cat# 17504-044
Fetal Bovine Serum (FBS)	Thermo Fisher Scientific	Cat# 16000-044
DMEM	Thermo Fisher Scientific	Cat# 11950-040
RPMI 1640	Thermo Fisher Scientific	Cat# 11875-093
Neurobasal	Thermo Fisher Scientific	Cat# 21103-049
DMEM/F-12	Thermo Fisher Scientific	Cat# 11330-032
KnockOut Serum Replacement	Thermo Fisher Scientific	Cat# 10828-028
NEAA	Thermo Fisher Scientific	Cat# 11140-050
GlutaMAX	Thermo Fisher Scientific	Cat# 35050-061
MEM Amino Acids Solution (EAA)	Thermo Fisher Scientific	Cat# 11130-051
Sodium pyruvate	Thermo Fisher Scientific	Cat# 11360-070
JNK Inhibitor VIII	Millipore	Cat# 420135
SB203580	Tocris	Cat# 1402
A-419259	Tocris	Cat# 3914
XAV939	Sigma-Aldrich	Cat# X3004
Bovine Albumin Fraction V (7.5% solution)	Thermo Fisher Scientific	Cat# 15260-037
M16	Sigma-Aldrich	Cat# M7292
Normal Donkey Serum	Jackson ImmunoResearch Laboratories	Cat# 017-000-121
Saponin	MP Biomedicals	Cat# 102855
Trypsin-EDTA (0.05%)	Thermo Fisher Scientific	Cat# 25300-054
TrypLE Express	Thermo Fisher Scientific	Cat# 12604-013
2-mercaptoethanol	Thermo Fisher Scientific	Cat# 21985-023
Pregnant mares' serum gonadotrophin (PMSG)	Prospec-Tany TechnoGene	Cat# HOR-272
Human chorionic gonadotrophin (HCG)	Sigma-Aldrich	Cat# CG10-1VL
Evans Blue	MP Biomedicals	Cat# 151108
Proteinase K	Thermo Fisher Scientific	Cat# AM2546
TRIzol	Thermo Fisher Scientific	Cat# 15596026
Versene	Lonza	Cat# 17711E
Accumax	Innovative Cell Technologies	Cat# AM105
Mineral oil	Sigma-Aldrich	Cat# M8410
Tyrode's Solution, Acidic	Sigma-Aldrich	Cat# T1788
Dnase	STEMCELL Technologies	Cat# 07900
Gelatin	Sigma-Aldrich	Cat# G1890
EAA (50×)	Gibco	Cat# 11130

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Streptomycin	Sigma-Aldrich	Cat# S6501
Sodium Penicillin G	Sigma-Aldrich	Cat# P3032
Ketamine	Putney	ANADA 200-055
Xylazine	Akorn	NADA 139-236
O.C.T. compound	SCIgen	Cat# 23730573
Critical commercial assays		
PrimeSTAR GXL DNA Polymerase	Clontech	Cat# R050B
Experimental models: Cell lines		
CF1 Mouse Embryonic Fibroblasts	Sciencell Research Laboratories	M7570-5
EPS cell	homemade	N/A
Experimental models: Organisms/Strains		
ICR female	The Jackson Laboratory	Stock No: 009122; RRID: IMSR_JAX:009122
Other		
u-Slide 8 well	ibidi	Cat# 80826
AggreWell™400 24-well plate, 5 pack	STEMCELL Technologies	Cat# 34415

MATERIALS AND EQUIPMENT

CDF12 medium		
Reagent	Final concentration	Amount
DMEM/F-12	n/a	400 mL
KnockOut Serum Replacement	20 %	100 mL
NEAA (100×)	1×	5 mL
GlutaMAX (100×)	1×	5 mL
2-mercaptoethanol (55 mM)	0.1 mM	0.91 mL
Total	**n/a**	**500 mL**

⚠ CRITICAL: Sterilize by filtering with a 0.22 mm filter. Store medium at 4°C and use within 1 month.

N2B27^{LCDM} (Yang et al., 2017b)		
Reagent	Final concentration	Amount
DMEM/F12 medium	n/a	240 mL
Neurobasal medium	n/a	240 mL
N2 supplement (100×)	0.5×	2.5 mL
B27 supplement (50×)	0.5×	5 mL
Glutamax (200 mM)	2 mM	5 mL
NEAA (100×)	1×	5 mL
2-mercaptoethanol (55 mM)	0.1 mM	0.91 mL
20% BSA or KnockOut Serum Replacement (optional)	0.1% or 5%	2.5 mL or 25 mL
hLIF (100 μg/mL)	10 ng/mL	50 μL
CHIR99021 (30 mM)	3 μM	50 μL
(S)-(+)-Dimethindene maleate (20 mM)	2 μM	50 μL
minocycline hydrochloride (20 mM)	2 μM	50 μL
Total	**n/a**	**500 mL**

⚠ CRITICAL: Sterilize by filtering with a 0.22 mm filter. Store medium at 4°C and use within 1 month.

EPSCM medium (Yang et al., 2017a)		
Reagent	Final concentration	Amount
CDF12 medium	n/a	500 mL
hLIF (100 μg/mL)	10 ng/mL	50 μL
CHIR99021 (30 mM)	3 μM	50 μL
PD0325901 (10 mM)	1 μM	50 μL
JNK Inhibitor VIII (40 mM)	4 μM	50 μL
SB203580 (10 mM)	10 μM	500 μL
A-419259 (3 mM)	0.3 μM	50 μL
XAV939 (50 mM)	5 μM	50 μL
Total	**n/a**	**500 mL**

⚠ CRITICAL: Sterilize by filtering with a 0.22 mm filter. Store medium at 4°C and use within 1 month.

ETS base medium

The ETS base medium is a 1:1 mix of N2B27 medium and the trophoblast stem cell base medium.

Reagent	Final concentration	Amount
RPMI 1640 medium	n/a	219 mL
DMEM/F12 medium	n/a	109.5 mL
Neurobasal medium	n/a	109.5 mL
FBS	10%	50 mL
Glutamax (200 mM)	2 mM	5 mL
NEAA (100×)	0.5×	2.5 mL
2-mercaptoethanol (55 mM)	0.1 mM	0.91 mL
Sodium pyruvate (100 mM)	0.5 mM	2.5 mL
N2 supplement (100×)	0.25×	1.25 mL
B27 supplement (50×)	0.25×	2.5 mL
Total	**n/a**	**500 mL**

⚠ CRITICAL: Sterilize by filtering with a 0.22 mm filter. Store medium at 4°C and use within 1 month.

KSOM medium (Biggers et al., 1997)		
Reagent	Final concentration	Amount
NaCl	95 mM	5.55 g
KCl	2.5 mM	0.186 g
KH_2PO_4	0.35 mM	0.0476 g
$MgSO_4 \cdot 7H_2O$	0.2 mM	0.0493 g
$NaHCO_3$	25 mM	2.100 g
$CaCl_2 \cdot 2H_2O$	1.71 mM	0.252 g
D-Glucose	5.56 mM	1 g
Sodium Lactate	10 mM	1.121 g

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Continued		
Reagent	Final concentration	Amount
Sodium Pyruvate	0.2 mM	0.022 g
EDTA	0.01 mM	0.00372 g
L-Glutamine	1 mM	0.146 g
BSA	0.4%	4 g
Sodium Penicillin G	60 mg/L	0.06 g
Streptomycin	50 mg/L	0.05 g
EAA (50×)	0.5×	10.0 mL
NEAA (100×)	0.5×	5 mL
H_2O	n/a	985 mL
Total	**n/a**	**1000 mL**

⚠ CRITICAL: Sterilize by filtering with a 0.22 mm filter. Store medium at 4°C and use within 1 month.

Alternatives: KSOM Embryo Culture (1×) is also available from Millipore (Cat# MR-020P-5D). Note that it contains only 0.1% BSA. Thus, we routinely supplement 0.3% BSA to achieve a final BSA concentration of 0.4%. In some experiments, we also used M16 (Sigma, M7292) to replace KSOM and obtained similar results.

Cell seeding medium		
Reagent	Final concentration	Amount
ETS base medium	0.5X	5 mL
KSOM medium	0.5X	5 mL
rhFGF4 (25 μg/mL)	12.5 ng/mL	5 μL
Heparin (1 mg/mL)	0.5 μg/mL	5 μL
ROCK Inhibitor Y-27632 (10 mM)	2 μM	2 μL
Chir99021 (30 mM)	3 μM	1 μL
Total	**n/a**	**10 mL**

⚠ CRITICAL: Use medium immediately after making.

Blastoid induction medium		
Reagent	Final concentration	Amount
ETS base medium	0.5×	5 mL
KSOM medium	0.5×	5 mL
rhFGF4 (25 μg/mL)	12.5 ng/mL	5 μL
Heparin (1 mg/mL)	0.5 μg/mL	5 μL
Chir99021 (30 mM)	3 μM	1 μL
A83-01 (5 mM)	0.5 μM	1 μL
BMP4 (50 μg/mL)	5 ng/mL	1 μL
Total	**n/a**	**10 mL**

⚠ CRITICAL: Use medium immediately after making.

Wide bore pipette tips

Wide bore pipette tips can be prepared by using a sterile blade or scissor to cut out a small proportion of the regular narrow bore pipette tips to make the opening bigger (Figure 1).

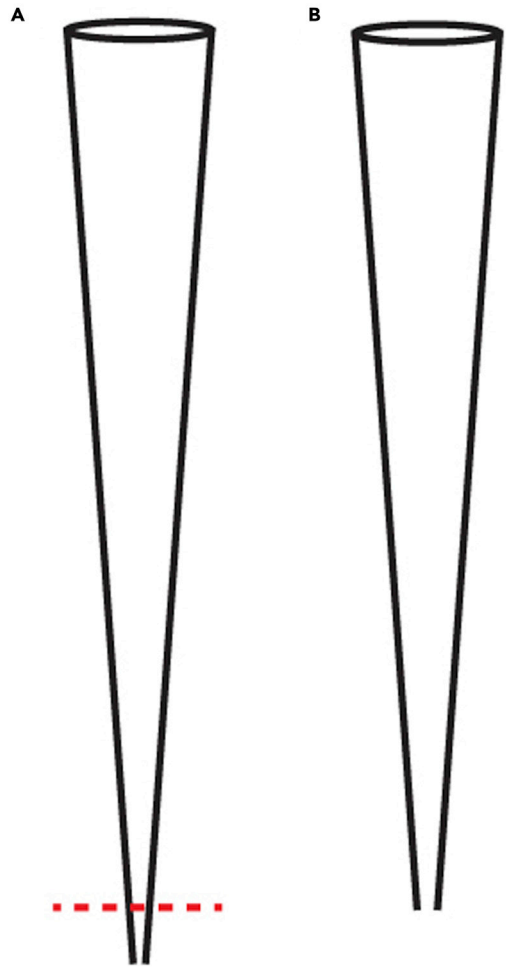

Figure 1. A diagram showing the preparation of wide bore tips

(A) Cutting the tip end of the normal pipette tips. The red line indicated the cut site.

(B) The look of the pipette tips after cutting off the end.

Alternatives: Wide bore pipette tips are available commercially, such as ART Wide Bore Filtered Pipette Tips (Thermo Scientific, 2069G).

STEP-BY-STEP METHOD DETAILS

Make single EPS cell suspension

Timing: ~2 h

The EPS cell culture is dissociated into single cells, followed with MEFs removal.

1. Dissociate EPS colonies on MEFs:
   a. Aspirate medium and wash cells with DPBS once.
   b. Add TrypLE (Thermo Fisher Scientific, 1 mL/10 cm^2 area) to cells and incubate the plate at 37°C for about 3 min.

Note: Check cells under a microscope to ensure that cells are mostly detached. If not, extend the incubation time.

c. Add CDF12 medium in an equal volume of TrypLE and pipette up and down several times to resuspend cells.
d. Centrifuge at 300 *g* for 5 min and remove supernatant.
e. Resuspend cells in 1 mL EPS medium.

2. Remove MEFs by differential attachment:
   a. Transfer cell resuspension to a gelatin-coated 6-well plate and incubate at 37°C for 1 h (MEF cells attached to the plate while EPS cells were still floating in suspension).

   Note: MEFs attach faster than EPS cells. After 1 h incubation, check the plate under a microscope to ensure that most MEFs are depleted from the suspension. MEF cells are easy to distinguish as they are much bigger than EPS cells.

   b. Collect cell suspension and filter it through a 45 μm cell strainer to eliminate cell aggregates.
   c. Count cells using the Bio-Rad TC-10 counter or a hemocytometer.

   Note: Bio-Rad TC10 counter requires a cell density at 1×10^5–5×10^6 cells/mL for optimal counting results. We usually count 2–3 times and calculate the mean as the readout.

   ⚠ CRITICAL: The cells should be healthy and surviving well at the end of the procedure. The percentage of live cells must be greater than 80% according to trypan blue staining. Troubleshooting 1

Aggregate EPS cells for blastoid formation

Timing: 5–7 days

Next, we seed the dissociated EPS cells into microwells for the formation of small cell aggregates and subsequently EPS-blastoids.

3. Calculate the volume for the required number of cells.

   Note: We routinely prepare cells enough for (N+1) wells (N is the number of wells to be seeded) to offset the pipetting loss.

   ⚠ CRITICAL: Cell seeding density is critical to the efficiency of blastoid formation. Typically, we seed an average of 5 cells/microwell in AggreWell 400. As each well of the AggreWell 400 24-well plate contains about 1,200 microwells, 6,000 cells are needed per well. For 6-well plate which contains about 7,000 microwells per well, 35,000 cells are needed. Also, depending on the accuracy of the cell count and the cell line difference, the optimal cell seeding number may vary. Therefore, we recommend testing a cell seeding density series (2 to 10 cells per microwell) to identify the optimal seeding density.

4. Prepare a sufficient amount of cell seeding medium as mentioned in materials and equipment.
5. Mix cells with medium, and pipette 500 μL into each well of the AggreWell 400 24-well plate or 2 mL for the AggreWell 400 6-well plate.
6. Shake the plate and spin down at 200 *g* for 2 min.

   Note: Shake the plate back and forth or from left to right. Never swirl it.

7. Check cells under a microscope to ensure that cells sit at the bottom of the microwells and are evenly distributed in all of the microwells.

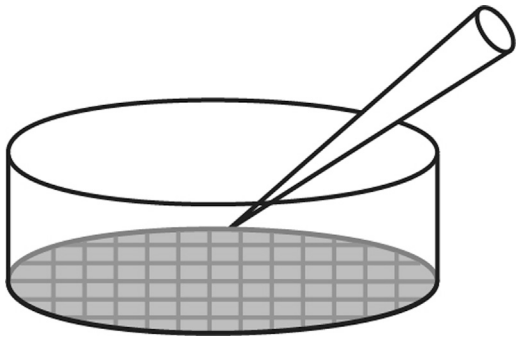

Figure 2. A diagram illustrating the placement of the tips for removing the medium and avoiding disturbance of the cells in the microwells

Note: If cells are not evenly distributed, pipette several times with a wide bore pipette tip to resuspend the cells and centrifuge again.

8. On the next day, check cell culture under the microscope. The cells in each microwell should form a compact aggregate at this time.

⚠ CRITICAL: Early signs of the compaction process can be seen as early as 4 h after cell seeding. If cells are still dispersed after 24 h of cell seeding, they are most likely dead. This can be due to excessive pipetting or over-digestion. Troubleshooting 2

9. Remove medium by tilting the plate slightly and aspirating medium with a P200 tip by gently placing the tip against the edge between the side and the bottom (Figure 2).
10. Replace with blastoid induction medium (see recipe in "materials and equipment", 500 μL for AggreWell 400 24-well plate).

Optional: Further medium change is optional. If the medium becomes acidic (yellow in color), or extended culturing is desired, additional medium change is necessary.

11. Check the culture daily to monitor the progress. EPS-blastoids usually emerge from Day 3 to Day 5. Troubleshooting 3 and 4

EPS-blastoid picking

Timing: 1–2 h

EPS-blastoids are identified and picked up for further experimentations or analyses. We identified the blastoids as having a cavitated sphere-like structure. The structures are round shaped or near round shaped with a diameter of 60–170 μm. These cavitated structures are easy to spot under a stereo microscope.

12. Using wide bore pipette tips to blow off the blastoid from the microwells by gently pipetting up and down.

Note: Gentle pipetting is strongly recommended to minimize blastoid breakage. The use of wide-bore pipette tips helps minimize the shearing pressure on EPS-blastoids during pipetting and maintains integrity.

13. Transfer the EPS-blastoid culture into a 6-well plate for picking. Troubleshooting 5.

Note: Alternatively, P35 dish or Nunc 4-Well dishes for IVF can be used.

14. Place the stereo microscope inside a biosafety cabinet and disinfect.
15. Place the plate containing the EPS-blastoid culture under the microscope.
16. Use a mouth pipette with a hand-pulled glass microcapillary pipettes to pick up EPS-Blastoids.

Note: Mouth pipette is routinely used to manipulate embryos. We used an aspirator tube assembly (Sigma-Aldrich, A5177) and attached a pulled glass microcapillary pipette. Note that the diameter of the opening in the glass pipette should be slightly larger than the maximum size of the embryo (~170 μm). Pre-wetting the glass pipette with 1% BSA in PBS helps reduce the chances of EPS-blastoids sticking to the glass.

Alternatives: Other pipetting equipment, such as the P10 or P200 pipette, should also be OK to use for picking up and washing blastoids. Pre-wetting the pipette tips with 1% BSA in PBS helps reduce the chances of EPS-blastoids to stick to the tips.

17. Place the pooled EPS-Blastoids into two drops of PBS successively for washing.

18. Use EPS-blastoid for immunostaining analysis or further culturing:
    a. Transfer the EPS-Blastoid into a drop of 4% PFA for fixation and further immunostaining analysis.

Pause point: Fixed EPS-Blastoids can be stored in PBS at 4°C for months.

    b. Transfer EPS-blastoids onto an ibidi μ-plate for further culturing using the IVC1/IVC2 or a similar culture system.

EPS-blastoids implantation

Timing: 2–3 h

Transfer manually picked EPS-blastoid into the mouse uterus for further experimentations or analysis.

19. Pick up EPS-blastoids manually and transfer them into a KSOM drop using a mouth pipette.
20. Anesthetized the ICR female surrogate (2–4 months old) at 2.5 days post coitum (dpc) with ketamine (Putney, 100 mg/kg, dissolved in saline at 10 mg/mL) and xylazine (Akorn, 16 mg/kg, dissolved in saline at 1.6 mg/mL).
21. Expose the uterine horn surgically and punctured it with a 27G needle.
22. Washes EPS-blastoids three times in KSOM drops.
23. Loaded EPS-blastoids into the pipette and transfer them to the uterine horn through the puncture.

Note: Around 20 EPS-blastoids were transferred into each uterine horn. The process of transfer was typically performed within 20–30 min per surrogate.

24. Sacrifice the surrogate and performed Caesarean section (C-section) at 6.5, 7.5, or 8.5 dpc, and dissected out the uterus. Troubleshooting 6.

Note: To stain the decidua, we inject 0.5% Evans Blue (MP Biomedicals, 151108) into the tail vein of the surrogate mice and wait for 15 min before starting the C-section.

25. Dissected deciduae out of the uterus, and dissect embryo-like structures out of the deciduae.
26. Fixed tissue samples with 4% PFA overnight (14–16 h) and embedded in the O.C.T compound.

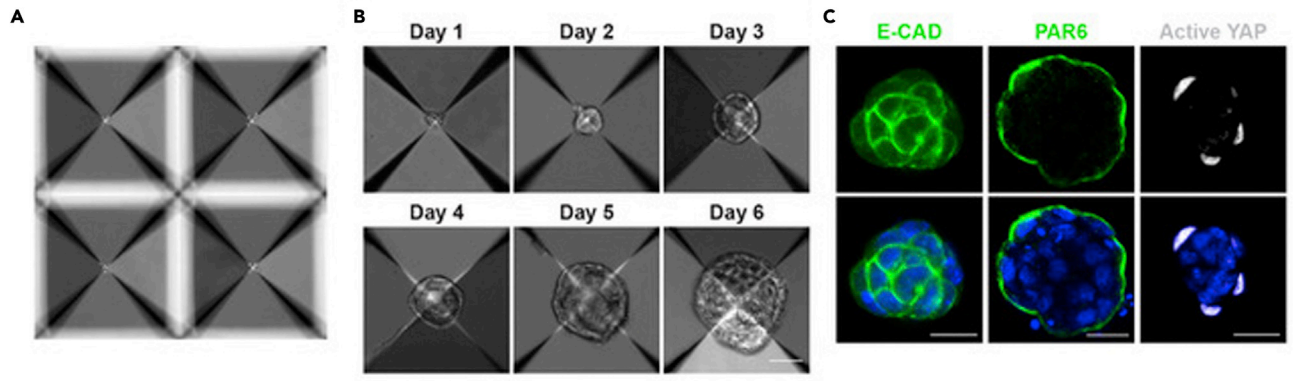

Figure 3. The progression of EPS-blastoids formation

(A) A representative image showing the cells immediately after seeding and centrifuging.

(B) Representative phase-contrast of EPS cell aggregates at the indicated time point show the formation of EPS-blastoids. Scale bar, 50 μm.

(C) Immunofluorescence staining for the expression of E-CAD in a day 1 aggregate, PAR6 in a day 2 aggregate, and active YAP in a day 2 aggregate. Scale bar, 20 μm.

Figure reprinted with permission from Li et al., 2019.

27. Cut Frozen sections (10 mm thick) using a microtome cryostat (Leica, model# CM1900-3-1).

Pause point: Sections can be stored at −20°C or lower for months.

28. Perform immunostaining on the sections.

EXPECTED OUTCOMES

Progression of EPS-Blastoid formation

We usually define the day of cell seeding as day 0 of the process (Figure 3A). Around 18–24 h after cell seeding (day 1), the EPS cells form compact aggregates (Figure 3B). The EPS cell aggregate will grow up and increase its size over time. Starting from day 3, some EPS cell aggregates become cavitated. These EPS-blastoids continued to enlarge, reaching an early blastocyst-like size at day 5 or day 6.

EPS-blastoid formation recapitulates some key cellular and molecular events characteristic of early preimplantation development(Rossant and Tam, 2009), including compaction (Wang et al., 2008), polarization (Chazaud and Yamanaka, 2016), and YAP subcellular localization (Kaneko and DePamphilis, 2013; Nishioka et al., 2009; Posfai et al., 2017; Rayon et al., 2014; Yagi et al., 2007). We can also check the occurrence of these events during the formation of EPS-blastoids (Figure 3C). On day 1, the cell adhesion protein E-cadherin and the tight junction protein ZO1 are highly expressed in these cell aggregates. On day 2, a fraction of cell aggregates (~33%) began to show PAR6 enrichment on the apical surface (Figure 3C), and on day 3, the majority of cell aggregates (~75%) are polarized. On day 2, YAP could be found in the nucleus of some cells at the periphery of the aggregate (Figure 3C). This YAP nuclear localization was observed in ~7% of aggregates. On day 3, ~27% of aggregates contained cells with nuclear localization of YAP (Figure 3C).

Staining of EPS-Blastoids

We usually stain EPS-blastoids with TE markers (CDX2, CK8, EOMES, etc), ICM/epiblast markers (OCT4, NANOG, SOX2, etc), and primitive endoderm markers (GATA6, GATA4, etc). The majority of EPS-blastoids (74%, n = 140) should express CDX2 on the outside layer of cells (Figure 4A) and have OCT4/NANOG/SOX2+ ICM-like compartments (Figure 4B). A fraction of the EPS-blastoid (21%, n = 112) will have a PE-like structure (GATA4/GATA6 +) (Figure 4C) intermingled with or on top of the epiblast-like cells (OCT4/NANOG/SOX2+).

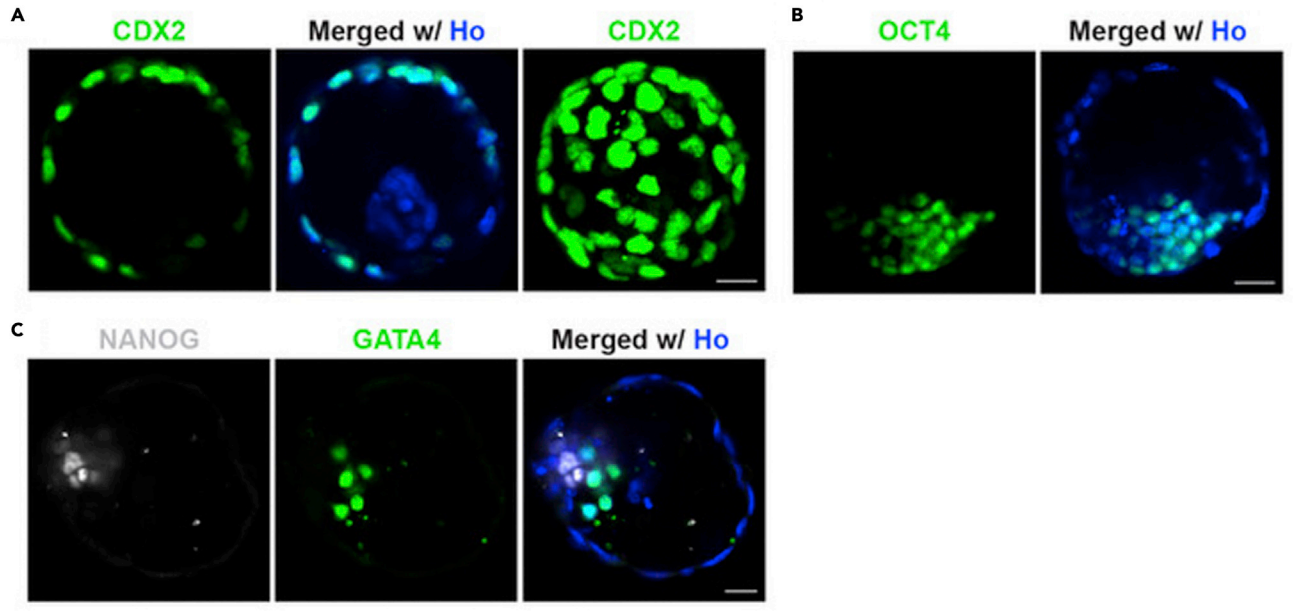

Figure 4. Immunofluorescence staining of EPS-blastoids at day 5 for the indicated markers

(A–C) Immunofluorescence staining of EPS-blastoids for expression of CDX2 (A), OCT4 (B), and Nanog and GATA4 (C), respectively. Scale bar, 20 μm. Figure reprinted with permission from Li et al., 2019.

EPS-blastoid-induced decidualization

Decidua is expected to form 4–6 days after the transfer of EPS-blastoids to the pseudopregnant uterus (Figure 5A). These EPS-blastoid-induced deciduae should stain positive with Evans blue dye (Figure 5B). After dissecting the decidua, we expect to see structures derived from EPS-blastoids inside. Compared to 6.5–8.5 dpc control embryos, the EPS-blastoid-derived structures might appear retarded or disorganized (Figures 5C and 5D). Immunostaining of these structures for the expression of the three lineages markers (e.g., OCT4 for epiblast, EOMES for ExE, GATA4 for VE) should reveal the presence of positive cells with these markers (Figures 5E–5G).

LIMITATIONS

The EPS-blastoid model recapitulates some events from pre-implantation to peri-implantation, including cell compaction, polarization, changes in subcellular YAP localization, lineage segregations, and implantation. Despite these similarities to blastocyst development, the formation of EPS-blastoid might not follow all the developmental steps as blastomeres develop into blastocysts and might rely on different signaling pathways. For example, Wnt signaling is dispensable for blastocyst development, but its blockage significantly reduces blastoid formation efficiency.

EPS-blastoids can implant into the uterus and induce decidualization. However, EPS-blastoids have very limited developmental potential beyond implantation *in vivo* and only form disorganized structures. In addition, blastoids generated using a variety of protocols developed by others (Kime et al., 2019; Rivron et al., 2018; Sozen et al., 2019) were also unable to develop into a fetus *in vivo*. It remains a challenge in the field to create fully functional synthetic blastocysts from cultured cells. Therefore, it might not be feasible now to use the EPS-blastoid to study the post-implantation developmental events *in vivo*.

TROUBLESHOOTING

Problem 1

Too many dead cells in the EPS single-cell suspension after dissociation and MEF removal (step 2).

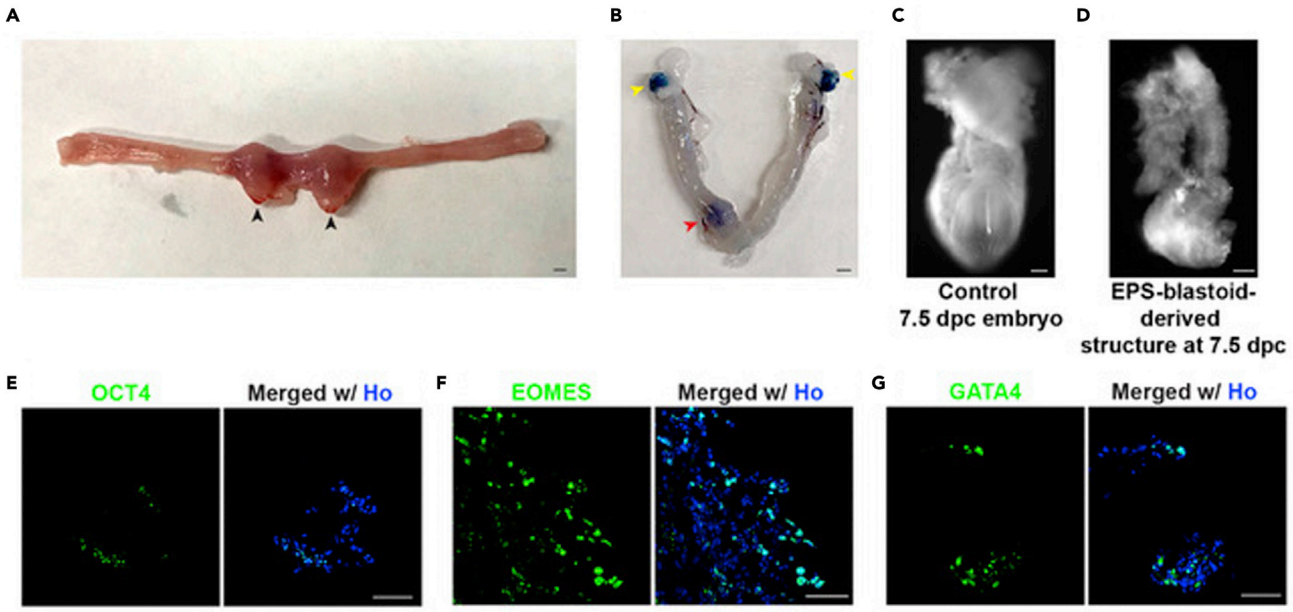

Figure 5. *In vivo* developmental potential of EPS-Blastoids

(A) Bright-field image showing the formation of decidua in the mouse uterus 5 days after EPS-blastoid transfer at 2.5 dpc. Black arrowheads indicate deciduae. Scale bars, 1 mm.

(B) Bright-field image of a mouse uterus 5 days after EPS-blastoid transfer at 2.5 dpc with Evans blue staining. The red arrowhead indicates a decidua. Yellow arrowheads denote the ovaries. Scale bars, 1 mm.

(C and D) Bright-field images of a control 7.5 dpc embryo (C) or an *in vivo* EPS-blastoid-derived structure recovered from decidua at 7.5 dpc (5 days after EPS- blastoid transfer) (D). Scale bars, 100 mm.

(E–G) Immunofluorescence staining of sections from an *in vivo* EPS-blastoid-derived structure recovered from decidua at 7.5 dpc (5 days after EPS-blastoid transfer) for OCT4 (E), EOMES (F), and GATA4 (G). Scale bars, 50 μm. Ho, Hoechst.

Figure reprinted with permission from Li et al., 2019.

Potential solution

EPS cell culture might not be in a good condition. If the cells are overgrown and all the nutrients are depleted, some cells will initiate apoptosis. Make sure not to grow the cells in too high density. Other possible reasons are 1) the incubation time for the digestion might be too long; 2) pipetting is too harsh; 3) The MEF removal process is too long. Try to stick to the recommended time and procedures.

Problem 2

Cells are dying, or very few aggregates remain in the plate after one or two days (step 8).

Potential solution

1) Cell seeding is too low. Better to try cell seeding gradient, for example, 1, 2, 3, 4, 5, up to 10 cells/microwell. Also, to ensure accurate pipetting of the cell suspension, dilute it to a suitable concentration before pipetting. 2) The medium might be old. Use fresh medium and reagents for making the cell seeding medium and blastoid induction medium. The critical components, such as B27 and FBS, should be tested in other cell cultures before using them for blastoid generation.

Problem 3

No blastoids or very few blastoids were generated in each well of the AggreWell plate (step 11).

Potential solution

1) EPS cell culture might not be in a good condition. The cells need to be healthy, exponentially proliferating, free of any types of contaminations, and without obvious differentiation. If the culture

shows early signs of deterioration, it is recommended to discard it and re-establish the culture from a frozen vial of cells. 2) The medium components are old or of low quality. Prepare fresh medium and use reagents from reliable vendors. 3) Cell seeding is too low or too high. Try a cell seeding series as stated above. 4) The ES or iPS cells have not been completely converted into the EPS state. Passage the ES or iPS cells in EPS condition for extra passages or check the quality of the components for making the EPS culture medium. The developmental potential of EPS cells can be tested by injecting cells into eight-cell embryos or blastocysts and examining the contribution of these cells in the embryonic and extra-embryonic lineages.

Problem 4

A large fraction of EPS-blastoids does not have the ICM compartment (step 11).

Potential solution

Normally, a small percentage (15%, n = 140) of EPS-blastoids does not have the ICM compartment. But if this type of trophosphere outnumbers the others, consider increasing the cell seeding density (5–10 cells/microwell) and also check the medium preparation.

Problem 5

EPS-Blastoids were broken or lost during transfer (step 13).

Potential solution

1) Collect the EPS-blastoid when they reach the optimal size (60–170 μm) to avoid manipulating oversized EPS-blastoids. 2) Practice gentle handling and use wide bore pipette tips. 3) Wet the tips or glass pipettes with 1% BSA solution before pipetting or mouth pipetting.

Problem 6

Lack of decidualization upon transfer or no recovery of any embryo-derived structures (step 24).

Potential solution

The decidualization rate is not high (~7% of transferred EPS-blastoids). Consider increasing the number of EPS-blastoids to be transferred to increase the chance of decidualization. Also, consider doing a co-transfer of blastocyst and EPS-blastoids. Embryo-derived structures at 6.5–8.5 dpc are very small and require delicate operations to dissect out. Consider practicing with normal embryos at E6.5–8.5 beforehand.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Juan Carlos Izpisua Belmonte (belmonte@salk.edu).

Materials availability

All unique/stable reagents generated in this study are available from the lead contact with a completed Materials Transfer Agreement.

Data and code availability

This study did not generate new data sets.

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AUTHOR CONTRIBUTIONS

R.L. and J.C.I.B. conceived the study, designed experiments, interpreted results, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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