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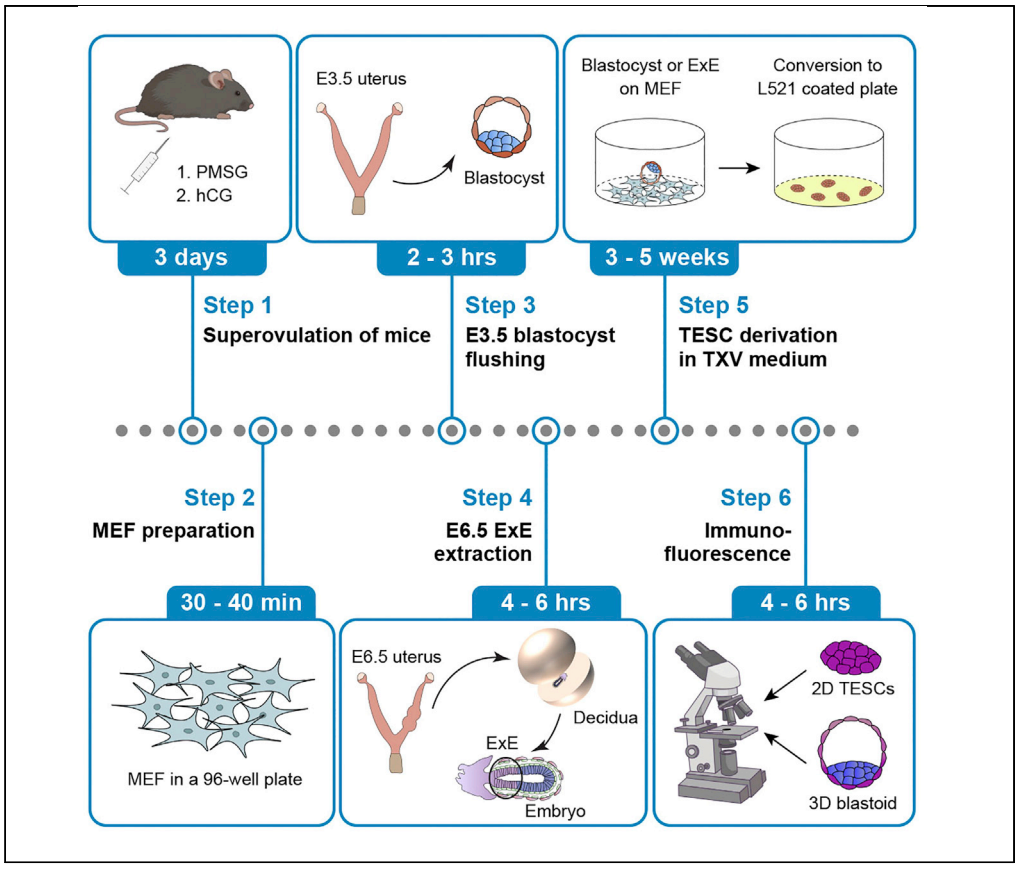
Protocol for capturing trophectoderm stem cells reflecting the blastocyst stage



Classically, culturing mouse blastocysts with FGF4/TGF-β1, two epiblast-secreted inducers, allows for deriving trophoblast stem cells that comprise fluctuating subpopulations reflecting both pre- and post-implantation stages. However, a more complete combination of inducers (adding LPA, IL11, BMP7, Activin A, 8-Br-cAMP) captures trophectoderm stem cells with enhanced transcriptomic similarity to the blastocyst trophectoderm and self-renewal, reduced differentiation, and increased potential to form blastoids and to instruct decidualization *in utero*, thus better reflecting the blastocyst.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Optimal inducers capture trophectoderm stem cells reflecting the blastocyst

Limits cell differentiation into post-blastocyst stages

Uses chemically defined, serum-free culture medium

Minimizes contamination of TESCs by other cell types

Seong & Rivron, STAR Protocols 4, 102151 June 16, 2023
https://doi.org/10.1016/j.xpro.2023.102151

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Protocol for capturing trophectoderm stem cells reflecting the blastocyst stage

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https://doi.org/10.1016/j.xpro.2023.102151

SUMMARY

Classically, culturing mouse blastocysts with FGF4/TGF-β1, two epiblast-secreted inducers, allows for deriving trophoblast stem cells that comprise fluctuating subpopulations reflecting both pre- and post-implantation stages. However, a more complete combination of inducers (adding LPA, IL11, BMP7, Activin A, 8-Br-cAMP) captures trophectoderm stem cells with enhanced transcriptomic similarity to the blastocyst trophectoderm and self-renewal, reduced differentiation. Also, the complete combination of inducers increased potential to form blastoids and to instruct decidualization *in utero*, thus better reflecting the blastocyst.

For complete details on the use and execution of this protocol, please refer to Seong et al.[1]

BEFORE YOU BEGIN

Stem cells enable studying development using 2D cultures or embryo models, e.g., blastoids. Since the first establishment of lines of TSCs using the epiblast inducer FGF4 and serum,[2] chemically-defined cultures comprising a more complete combination of inducers (FGF4, TGF-β1/Activin) have proved sufficient to maintain proliferative and self-renewing TSCs *in vitro*.[3,4] However, it is well assessed that such TSCs don't capture a restricted developmental stage but rather comprise several subpopulations reflecting the blastocyst trophectoderm (pre-implantation stage), the extra-embryonic ectoderm (ExE, the post-implantation derivative of the polar trophectoderm that contains the placental progenitors), and more differentiated trophoblasts as well.[1,5–10]

We proposed that this cellular heterogeneity observed in TSCs cultures teaches us that suboptimal exposure to epiblast inducers permits fluctuations of these subpopulations facilitating the escape from the self-renewing state and the differentiation of trophoblasts that is necessary for development.[1] We also proposed that a more complete and optimal combination of blastocyst-stage epiblast-secreted inducers captures Trophectoderm Stem Cells (TESCs) better reflecting the blastocyst trophectoderm.[1] These inducers prevent developmental progression by (1) enhancing the transcriptomic similarity to the trophectoderm and (2) repressing the gene expression associated with differentiation, (3) thus enhancing self-renewal and (4) the trophoblasts potential to recapitulate features of trophectoderm epithelial morphogenesis and to instruct the uterus to decidualize, while (5) retaining trophoblasts potential to rapidly differentiate and chimerize the ExE. Based on these criteria, we concluded that TESCs overall better reflect the blastocyst trophectoderm.[1]

Mouse blastoids are blastocyst models initially generated by combining TSCs and embryonic stem cells (ESCs).[11] Blastoids, like blastocysts, arise because of ESC-secreted inducers that direct TSCs to

undergo epithelial cyst morphogenesis while enriching them in trophectoderm mRNA transcripts.[11] Here, we propose that forming blastoids using TESCs allows us to start with a more developmentally matched state, and hence replicate aspects of blastocyst development more closely. It allowed us to show that inducers regulate CDX2 and the secretion of WNT6/7B in trophoblasts that contribute to both trophectoderm development and uterus decidualization.[11]

Here, we describe a protocol to efficiently derive TESCs from E3.5 blastocysts and E6.5 conceptus (95.7% and 83.3% of efficiency, respectively) without contamination of other cell types (e.g., extra-embryonic endoderm stem (XEN) cells). Chemically-defined culture conditions avoiding the use of serum in the medium limits various unknown (batch) effects as well. Because the trophoblasts progenitors are plastic[12,13] and maintained during the post-implantation stages by a niche created by dynamic interactions at the epiblast/trophoblast interface,[14] both TSCs and TESCs can be derived not only from the blastocyst but also from the E6.5 ExE. We hope that a detailed explanation of E6.5 embryo dissection will help not only to learn how to derive TESCs, but also to dissect and obtain ExE samples for early embryology research. The derivation of TESCs that more closely resemble the blastocyst trophectoderm enables research on the biological pathways and genetic mechanisms regulating early embryonic development and the implantation into the uterus.

Institutional permissions

All animal experiments were conducted using 12–16 week-old female mice on 129/Sv, FVB/N, C57BL/6J and B6CBAF1 background (F1 of CBA ♂ × C57BL/6J ♀), except MEF preparation (DR4 background). Mice were maintained at the research Institute of Molecular Biotechnology, Austrian academy of Sciences/Institute of Molecular Pathology (IMBA/IMP) animal house. All animal experiments were approved by the IMBA/IMP animal house and performed in accordance with the guidelines of the institution. Researchers will need to acquire permissions from the relevant local institutions before performing these experiments.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-CDX2	Abcam	ab76541
anti-TBR2/EOMES	Abcam	ab23345
anti-OCT3/4 (c-10)	Santa Cruz Biotechnology	sc-5279
anti-NANOG	Abcam	ab80892
anti-rabbit donkey IgG 647	Invitrogen	A31573
anti-mouse donkey IgG 568	Invitrogen	A10037
Hoechst	Invitrogen	H3570
Chemicals, peptides, and recombinant proteins		
DMEM/F-12	In-house	N/A
L-ascorbic acid 2-phosphate (ASAP)	Sigma	A8960
Gibco™ Insulin Transferrin Selenium (ITS-G)	Gibco	12097549
GlutaMAX	Gibco	35050038
Sodium pyruvate	Gibco	11360070
HEPES	In-house	N/A
β-Mercaptoethanol	Gibco	31350010
Penicillin/streptomycin	Sigma	P0781
MEM non-essential amino acid (NEAA)	Gibco	11140035
Human FGF-4	R&D system	235-F4
Human TGF-β1 (HEK293 derived)	PeproTech	100-21
Heparin	Sigma	H3149
Human/murine/rat activin A (CHO derived)	PeproTech	120-14P

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Murine IL-11	PeproTech	220-11
human BMP7	PeproTech	120-03p
8-Br-cAMP	BIOLOG	B 007-500
1-Oleoyl lysophosphatidic acid (LPA)	Tocris	2256236
Y-27632	MedChem Express	HY-10583
(Adv.) DMEM/F-12	Gibco	12634010
Neurobasal medium	Gibco	21103049
N-2 supplement	Gibco	17502048
B-27 supplement	Gibco	A1895602
CHIR 99021	MedChem Express	HY-10182
PD 0325901	MedChem Express	HY-10254
LIF (ESGRO)	Merck	ESG1107
BSA (35%)	Sigma	A7409
DMEM high glucose	In-house	N/A
Fetal bovine serum (FBS)	Sigma	F7524
Phosphate buffered saline (PBS)	In-house	N/A
Donkey serum	Sigma	D9663-10ML
Gelatin solution	Sigma	G1393
EmbryoMax M2 medium	Millipore Sigma	MR-015P-D
Human recombinant Laminin 521	Biolamina	LN521-05
Accutase	BioLegend	423201
TrypLE Express Enzyme (1×), no phenol red	Gibco	12604039
Pregnant Mare Serum Gonadotropin (PMSG)	Hölzel Diagnostika	OPPA01037
human chorionic gonadotropin (hCG)	MSD	Chorulon 1500 IU
Tyrode's acid solution	Sigma	T1788
Triton x-100	Sigma	X100-100ml
Penicillin/streptomycin	Gibco	15140122
Experimental models: Cell lines		
F4-GFP TSCs	Gift obtained from Hospital for Sick Children, Toronto, Ontario, Canada (J. Rossant lab)	N/A
V6.5 ESCs	Gift obtained from Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada (A. Nagy lab)	N/A
V6.5 ESCs-H2B-mTurquoise	This paper (plasmid transfection on the V6.5 ESCs)	N/A
LB-A5 TESCs	This paper (F1 of CBA ♂ × C57BL/6J ♀, or B6CBAF1 wild-type *Mus musculus*). Cell lines come from E3.5 female embryo)	N/A
Experimental models: Organisms/strains		
Mouse_129/Sv (*Mus musculus*)	Janvier Labs	RRID:MGI:2160041
Mouse_FVB/N (*Mus musculus*)	The Jackson Laboratory	RRID:IMSR_JAX:001800
Mouse_C57BL/6J (*Mus musculus*)	The Jackson Laboratory	RRID:IMSR_JAX:000664
Mouse_CBA (*Mus musculus*)	The Jackson Laboratory	RRID:IMSR_JAX:000656
Mouse_B6CBAF1 (*Mus musculus*)	CBA ♂ × C57BL/6J ♀	N/A
Recombinant DNA		
PB-CAG-H2B-mTurquoise	Gift obtained from Max Perutz Labs, Vienna, Austria (C. Bücker lab)	N/A
Software and algorithms		
Adobe Illustrator 2022	Adobe	https://www.adobe.com/products/illustrator.html
Adobe Photoshop 2022	Adobe	https://www.adobe.com/nl/products/photoshop.html
Fiji	NIH	https://imagej.net/Fiji
Excel	Microsoft	https://www.microsoft.com/nl-nl/microsoft-365/excel

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Others		
Evos M7000	Invitrogen	AMF7000
Dumont #5 Forceps - Biologie/Ceramic Coated	Fine Science Tools	11252-50
Fine scissors - Martensitic Stainless Steel/Straight/10.5cm	Fine Science Tools	14094-11
Holders for standard retransfer & handling pipettes (mouth pipette)	BioMedical Instruments	N/A
Handling pipettes (135–144 μm)	BioMedical Instruments	N/A
35 mm culture dish	FALCON	353001
60 mm culture dish	FALCON	353004
35 × 10 mm dish with 4 inner rings	Greiner bio-one	627 170
Micro-Fine+ Insulin syringe 0.5 mL, 29G	BD	324824
Eclipse needle with smartslip technology, 27G × 3/4	BD	305889
Syringe without needle	TERUMO	SS+01T1
μ-slide 4 well-ibiTreat	Ibidi	80426
Micro-insert 4 wells in μ-Dish 35 mm high	Ibidi	80406
15 mL tube	Falcon	352096
96-well culture plate	Thermo Scientific	167008
48-well culture plate	Eppendorf	0030723112
12-well culture plate	Thermo Scientific	150628
6-well culture plate	Thermo Scientific	140675

MATERIALS AND EQUIPMENT

MEF medium

Reagent	Final concentration	Amount
DMEM high glucose	N/A	450 mL
FBS	10%	50 mL
MEM NEAA (100×)	1% (1×)	5 mL
Penicillin/streptomycin (100×)	1% (1×)	5 mL
Total	**N/A**	**510 mL**

Store at 4°C for up to 3 months.

Alternatives: Other antibiotics can be used in place of penicillin/streptomycin.

Basal TX medium

Reagent	Final concentration	Amount
DMEM/F-12	N/A	450 mL
ASAP (200 mM)	200 μM	0.5 mL
ITS-G (100×)	2% (1×)	10 mL
Sodium Pyruvate (100 mM)	1 mM	5 mL
GlutaMAX (100×)	1% (1×)	5 mL
HEPES (1 M)	10 mM	5 mL
β-mercaptoethanol (50 nM)	100 pM	1 mL
Penicillin/streptomycin (100×)	1%	5 mL
Total	**N/A**	**480 mL**

Store at 4°C for up to 3 months.

TXV medium		
Reagent	Final concentration	Amount
Basal TX	N/A	49.7 mL
FGF-4 (25 μg/mL)	25 ng/mL	50 μL
TGF-β1 (10 μg/mL)	2 ng/mL	10 μL
Heparin (1 mg/mL)	1 μg/mL	50 μL
Activin A (50 μg/mL)	50 ng/mL	50 μL
IL-11 (50 μg/mL)	50 ng/mL	50 μL
BMP7 (25 μg/mL)	25 ng/mL	50 μL
8-Br-cAMP (1 M)	200 μM	10 μL
LPA (5 μM)	5 nM	50 μL
Total	**N/A**	**50 mL**
Store at 4°C for up to 2 weeks.		

Note: Do not add 8-Br-cAMP and LPA for the storage. They need to be added to the TXV medium freshly to ensure their optimal activity.

TXV medium for cell passaging		
Reagent	Final concentration	Amount
TXV medium	N/A	5 mL
Y-27632 (2 mM)	2 μM	5 μL
Total	**N/A**	**5 mL**
Use it immediately after making it.		

Note: Y-27632 (Rock inhibitor) need be added when you passage cells for successful attachment of TSCs. No need to add Y-27632 when changing medium for already attached TSCs.

Wash buffer		
Reagent	Final concentration	Amount
DMEM/F-12	N/A	450 mL
HEPES (1 M)	10 mM	4.5 mL
BSA (35%)	0.1%	1.29 mL
Total	**N/A**	**456 mL**
Store at 4°C for up to 3 months.		

Basal B27N2 medium		
Reagent	Final concentration	Amount
DMEM/F-12	48.15%	120.4 mL
Neurobasal medium	48.15%	120.4 mL
N-2 supplement (100×)	0.5% (0.5×)	1.25 mL
B-27 supplement (50×)	1% (1×)	2.5 mL
GlutaMAX (100×)	1% (1×)	2.5 mL
HEPES (1 M)	10 mM	2.5 mL
β-mercaptoethanol (50 nM)	100 pM	0.5 mL
Total	**N/A**	**250 mL**
Store at 4°C for up to 1 month / at −20°C for up to 6 months.		

2i-Lif medium		
Reagent	Final concentration	Amount
Basal B27N2 medium	N/A	49.5 mL
Lif (10^5 unit)	10^3 unit	0.5 mL
PD 0325901 (10 mM)	1 μM	5 μL
CHIR 99021 (10 mM)	3 μM	15 μL
Total	**N/A**	**50 mL**
Store at 4°C for up to 1 week.		

Alternatives: Lif (final 20 nM).

PMSG		
Reagent	Final concentration	Amount
PMSG (1,000 unit)	5 unit / 100 μL	(1,000 unit)
PBS	N/A	20 mL
Total	**N/A**	**20 mL**
Store at −20°C for up to 6 months / at 4°C for up to 1 month.		

hCG		
Reagent	Final concentration	Amount
hCG (1,500 unit)	5 unit / 100 μL	(1,500 unit)
PBS	N/A	30 mL
Total	**N/A**	**30 mL**
Store at −20°C for up to 6 months. Use it immediately after defrosting.		

Laminin 521		
Reagent	Final concentration	Amount
Laminin 521	5 μg / mL	5 mL (500 μg)
PBS (with Ca^{2+}, Mg^{2+})	N/A	95 mL
Total	**N/A**	**100 mL**
Store at −20°C for up to 6 months. Use it immediately for plate coating after defrosting.		

Note: Make aliquots and freeze them so you can defrost only few vials when you need them.

Blocking buffer for immunofluorescence		
Reagent	Final concentration	Amount
PBS	N/A	9.13 mL
Donkey serum	3%	0.3 mL
35% BSA	2%	0.571 mL
Triton x-100	0.1%	10 μL
Total	**N/A**	**10 mL**
Store at 4°C for up to 1 month.		

Alternatives: You can change concentrations of serum (2%–10%), BSA (2%–5%), and Triton x-100 (0.1%–0.5%) for the optimalization of your experiments.

Alternatives: Goat serum can replace donkey serum if you do not use primary antibodies from goat and you use goat origin secondary antibodies.

0.1% PBS-T

Reagent	Final concentration	Amount
Triton x-100	0.1%	1 mL
PBS	N/A	1,000 mL
Total	**N/A**	**1,000 mL**

Store at 20°C–25°C, and there is no specific expire date.

0.3% PBS-T

Reagent	Final concentration	Amount
Triton x-100	0.3%	300 μL
PBS	N/A	100 mL
Total	**N/A**	**100 mL**

Store at 20°C–25°C, and there is no specific expire date.

mES medium

Reagent	Final concentration	Amount
DMEM high glucose	86.9%	217.5 mL
FBS	10%	25 mL
GlutaMAX (100×)	1% (1×)	2.5 mL
MEM NEAA (100×)	1% (1×)	2.5 mL
HEPES (1 M)	1% (10 mM)	2.5 mL
β-mercaptoethanol (50 nM)	0.1% (50 pM)	0.25 mL
Total	**N/A**	**250 mL**

Store at 4°C for up to 1 month / at −20°C for up to 6 months.

Blastoid mES medium

Reagent	Final concentration	Amount
mES medium	97%	9.7 mL
Lif (10^5 unit)	1.5 × 10^3 unit	150 μL
Penicillin/streptomycin	1.5%	150 μL
Total	**N/A**	**10 mL**

Use it immediately after making it.

Alternatives: Lif (final 30 nM).

Blastoid TX medium

Reagent	Final concentration	Amount
Basal TX	96%	9.6 mL
MEM NEAA (100×)	1.5% (1.5×)	150 μL
Penicillin/streptomycin	1.5%	150 μL

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Reagent	Final concentration	Amount
Y-27632 (20 mM)	30 μM	15 μL
8-Br-cAMP (1 M)	1.5 mM	15 μL
Heparin (1 mg/mL)	1.5 μg/mL	15 μL
FGF4 (25 μg/mL)	37.5 ng/mL	15 μL
TGF-β1 (10 μg/mL)	22.5 ng/mL	22.5 μL
IL-11 (50 μg/mL)	45 ng/mL	9 μL
CHIR 99021 (10 mM)	4.5 μM	4.5 μL
Total	N/A	10 mL

Use it immediately after making it.

Stereo microscope

Leica M80 Stereo Zoom Microscope 7.5×–60×.

Alternatives: Any stereo microscopes capable of using magnifications in the 10×–50× range should be fine.

Fluorescence microscope

Spinning disk confocal microscope based on an Olympus IX3 Series (IX83) inverted microscope, equipped with a dual-camera Yokogawa W1 spinning disk (SD).

Alternatives: Any fluorescence microscope capable of using 3–4 fluorescence filters would be fine.

STEP-BY-STEP METHOD DETAILS

Superovulation of mice

Timing: 3 days (30–60 min for each hormone injection)

This step describes how to prime the female mice with hormones to induce superovulation. It helps to get many embryos (10–30 blastocysts per mouse) on a given date.

1. Prepare 5 week-old or 12–16 week-old female mice (see problem 1 under troubleshooting).
2. Defrost the PMSG stock on ice.
3. Using an insulin syringe, inject 5–6 units of PMSG (100–120 μL) by intraperitoneal (i.p.) injection between 14:00–15:00 (day -2).
4. 46–48 h after PMSG injection, defrost the hCG stock on ice just before use.
5. Inject 5–6 units of hCG (100–120 μL) by i.p. injection between 13:00–14:00 (day 0).

Note: After defrosting, the hormones should be kept on ice while preparing for the injection.

Note: PMSG can be stored up to 1 month in a 4°C fridge, but we do not recommend storing the remaining hCG in a 4°C fridge because of a sharp decrease in hormone activity during storage.

6. Place the females into the stud male cages around 18:00–20:00 (day 0).

Alternatives: It is fine to start the breeding immediately after hCG injection, if necessary.

7. Next morning (07:00–09:00), check for the presence of a plug on the females' vagina (day 1, E0.5) (See problem 1 under troubleshooting).

Preparation of irradiated mouse embryonic fibroblasts (MEFs)

Timing: 30–40 min

This step describes how to prepare MEF plates before the main experiment of derivation. One or two days before E3.5 blastocyst flushing or E6.5 conceptus dissection, prepare a 96-well cell culture plate with gamma ray-irradiated MEF.

Alternatives: Gamma ray-irradiation can be replaced by Mitomycin C (MMC) treatment. Treat MEF with MMC (10 μg/mL in MEF medium) for 1–2 h before your experiment (Fisher Scientific). Remove the MMC and wash the MEF several times with PBS to ensure that no MMCs remain.

8. Prepare MEFs by purchasing commercial one or by extracting them from mouse embryos according to established protocols.[15]

Note: Although the mouse origin of MEFs might vary for each laboratory situation and protocols, we have achieved better results when using MEFs isolated from E13.5 DR4 rather than C57BL/6J mouse strains.

9. Coat each well of a 96-well cell culture plate with 50 μL of 0.1% gelatin in PBS, and incubate the plate at 37°C for at least 5 min.

Note: Plates including gelatin can be stored in the incubator for long periods so as to maintain a stock ready-to-go.

10. Prewarm the MEF medium or the washing buffer at 37°C in a water bath or at 20°C–25°C in a hood.
11. Take the MEF stock vials from liquid nitrogen and place them into the 37°C water bath until only a small ice clump is left.

Alternatives: You can also add 1–2 mL of pre-warmed medium into each MEF vial, and defrost them through gentle pipetting. However, shear stress can decrease cell viability.

12. Transfer the MEFs into the 10 mL of pre-warmed MEF medium or washing buffer in a 15 mL tube.
13. Centrifuge the MEFs at 200 g for 4 min.
14. Remove the supernatant from the tube, and tap the pellet several times with the remaining washing buffer to roughly dissociate it.
15. Resuspend the MEF pellet with the pre-warmed MEF medium.
16. Remove the gelatin solution from the 96-well plate.
17. Add 20,000–25,000 MEF cells per well and incubate the plate in a humidified incubator at 37°C with 5% CO_2.

Note: Defrost MEFs 1 day before use, for example the morning before, and check the attachment, quality, and confluency of the cells, for example before leaving on the same day. Eventually re-adjust the confluency by defrosting another vial if attachment and/or viability is lower than expected.

⚠ CRITICAL: For the blastocyst or ExE culture (passage 0) and for the first passage, MEFs need to be exposed to serum-free TXV medium for a relatively long time (around 1 week), which is challenging for them, as they can easily detach from the plate. To compensate, the confluency of MEFs should be higher than in other experiments (e.g., ESCs culture), and medium change should be performed very carefully (See problem 2 under troubleshooting).

Preparation of E3.5 blastocysts

Timing: 2–3 h

This step describes how to flush E3.5 blastocysts from the uterus of plug positive pregnant female mice.

18. Change the medium of MEF coated 96-well plate from MEF medium to 200 μL of TXV medium. Place them back in the humidified incubator (37°C with 5% CO_2) to prewarm the TXV medium for about 1 h before the deposition of the blastocysts or ExE tissues.

Note: LPA and cAMP should be added freshly to the TXV medium to ensure optimal activity.

19. Sacrifice the plug positive female mice 3.5 days after plugging (day 4; 3 days after plug check).

Note: Theoretically, for E3.5 blastocysts, females need to be sacrificed around 09:00, considering that mating and fertilization happens rapidly after gathering the males and females. However, for this experiment, the time point is not so critical because morula or early blastocysts will develop to the full-blown blastocyst stage *in vitro*, subsequently allowing for the derivation of TESCs.

20. Dissect uterus with the ovary and the upper cervix, and place them in PBS.

⚠ CRITICAL: In order not to crush the blastocysts, do not directly grab the uterus with forceps but rather use the ovary or cervix to handle the organs. One can easily distinguish the cervix from the uterus based on color (cervix: relatively white, while the uterus is pink or reddish. See Figures 1A and 1B).

Note: Although pregnant females tend to have a more vascularized uterus, it is difficult to evaluate whether the female is pregnant or not based on the uterus morphology. Not to lose blastocysts, take and flush every uterus.

21. After a brief wash with PBS, remove the fat tissues containing blood vessels that are on the mesometrium region of the uterus (Figure 1A).
22. Prepare one drop of M2 medium in the center of a 35 mm dish and in a 4-ring well plate (Figure 1C).

Note: We recommend you use a 35 mm dish rather than 60 mm or 100 mm dishes since it is easier to find the blastocysts if we use smaller dishes.

Note: One well of a 4-ring well plate can contain 100–120 μL of M2 medium.

23. Prepare a syringe filled with M2 medium.
24. Cut out the cervix from the uterus and the center of two horns of the uterus (Figure 1B). You might also cut ovaries. Put one horn in the M2 medium of the 35 mm dish.

Note: It is not necessary to remove the ovaries. They might help in identifying the direction of the uterus.

25. Insert the syringe needle into the ovary-side of the uterus horn and hold the tip of the needle and the horn together with forceps to prevent the needle from detaching from the horn during the flushing (Figures 1D and 1E) (See problem 3 under troubleshooting).

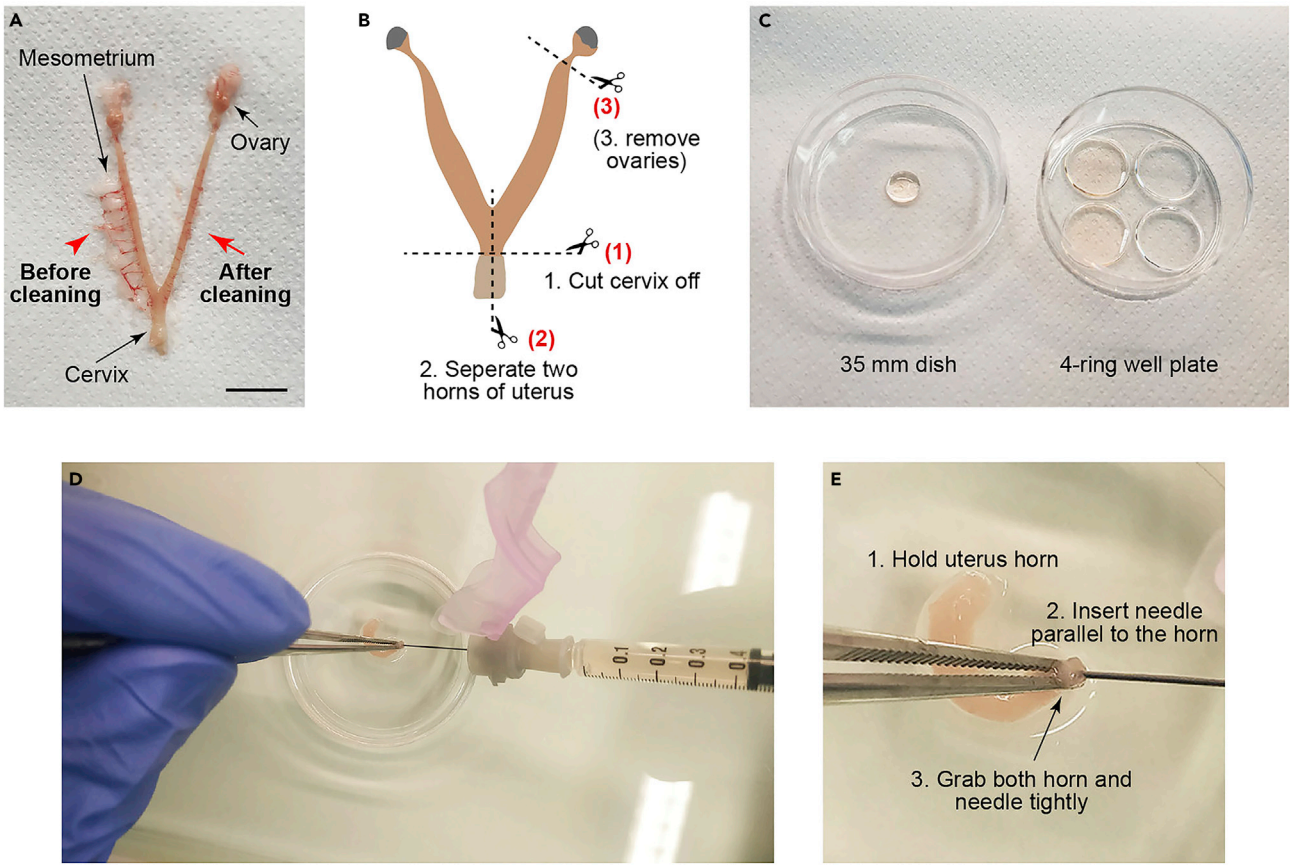

Figure 1. Dissection and flushing of E3.5 uterus

(A) E3.5 uterus. Cervix and uterus are distinguishable by colors. Scale bar: 1 cm.

(B) Procedures how to dissect E3.5 uterus for blastocyst flushing. Removal of ovaries is optional.

(C) M2 medium on a 35 mm dish and a 4-ring well plate.

(D and E) (D) Insertion of needle into one horn of E3.5 uterus, and (E) magnified image. The inserted needle needs to be tightly held by forceps. Related to steps 20–26.

26. By slowly pushing 300–500 μL of M2 medium, flush the uterus.
27. Repeat steps 24–26 for the remaining uterus.

Note: Use a new 35 mm dish once the dish has more than 2–3 mL of flushed M2 medium. After several flushings, both the presence of many debris and the thickness of the medium layer make it difficult to find the blastocysts.

28. Prepare the mouth pipette and fill up the capillary with M2 medium.
29. Using the mouth pipette, select the blastocysts using a stereo microscope and transfer them to a ring of a 4-ring well plate (See problem 4 under troubleshooting).

Note: In most cases, you will find sphere-shaped early and late blastocysts, but also some arrested cleavage-stage embryos, some morula, and few oval shaped, elongated or crushed late blastocysts. Such deformed late blastocysts are also good for TESC derivation (Figure 2A).

30. To remove the red blood cells and debris present in the medium, wash the morulae and blastocysts 2 times with M2 medium by transferring them from one ring well to another.

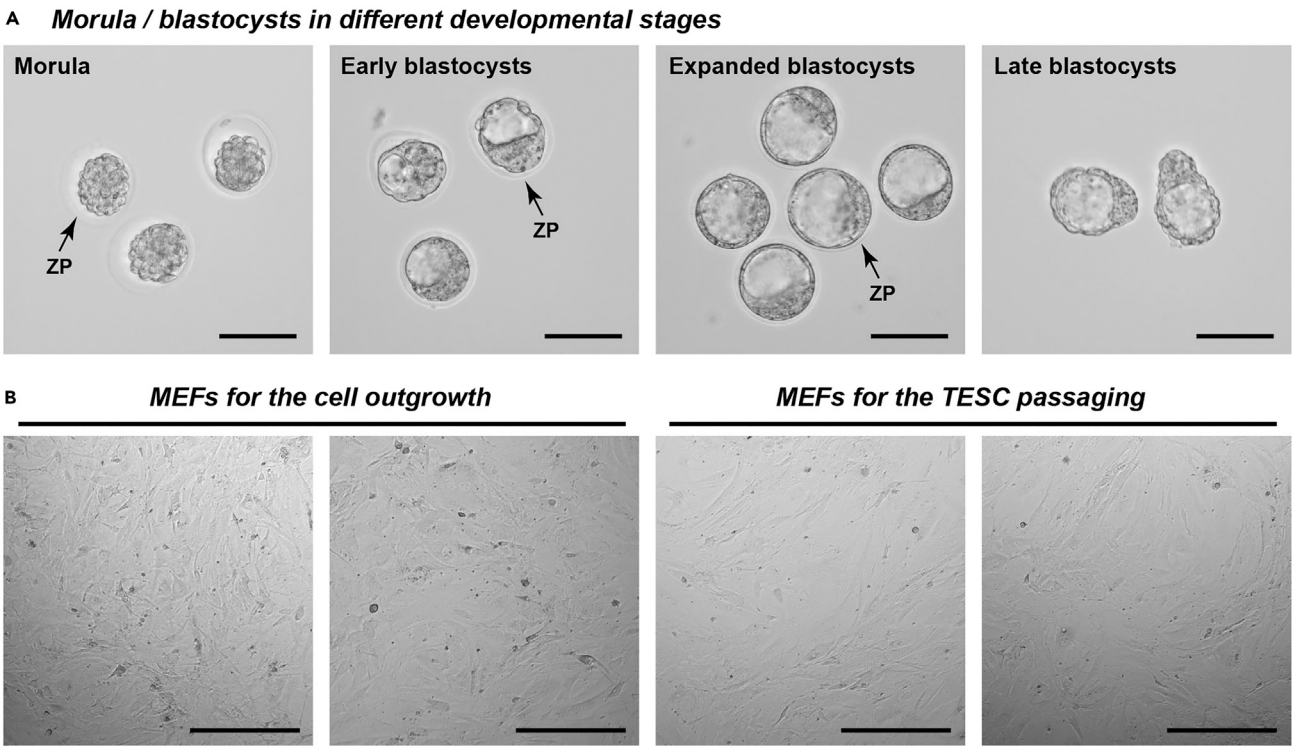

Figure 2. Preparation of MEF and blastocysts

(A) Examples of Morula and blastocysts in different developmental stages. After hatching, late blastocyst does not have ZP. Scale bar: 100 μm. ZP: zona pellucida.

(B) Examples of MEFs for the cell outgrowth and passaging. MEF should be highly confluent for the cell outgrowth. Scale bar: 300 μm. Related to steps 29–32.

31. Prepare the mouth pipette and fill the bended capillary up with TXV medium.
32. Use the mouth pipette to take the morulae and blastocysts and place one per well in the center of one well of MEF coated 96-well plate and including prewarmed TXV medium. Incubate the plates in a humidified incubator at 37°C with 5% CO_2.

Note: Check MEFs again if the MEFs are highly confluent (Figure 2B, left). After 2nd passage, one can use less confluent MEFs (Figure 2B, right; step 64).

Note: Removal of the Zona pellucida (ZP) is not necessary since most blastocysts will hatch from ZP in a few days after settling down onto the MEF. If you want, you can remove the ZP using a Tyrode's acid solution before placing them in the wells of the MEF coated 96-well plate. Make few drops of Tyrode's acid solution on an any petri dish (30–40 μL per drop). Transfer blastocysts to the drop using a mouth pipette, and suck the blastocysts up before they fall down to the bottom of the dish since blastocysts become very sticky after removal of the ZP. Once a blastocyst attaches to the dish, you cannot use it anymore. Repeat sucking and blowing blastocysts 5 to 10 times in a Tyrode's acid drop. Transfer the blastocysts to another drop of Tyrode's acid solution, and repeat again sucking and blowing blastocysts 5 to 10 times. To prevent losing them, wash them immediately and several times using 0.4% of BSA in PBS using a mouth pipette previously filled up with 0.4% of BSA in PBS but not TXV medium, and then transfer them into the wells of the MEF coated 96-well plate.

⚠ CRITICAL: Do not disturb the 96-well plate for at least 2 days as the initial adhesion process should not be disturbed.

Preparation and dissection of E6.5 conceptus

Timing: 4–6 h

This step describes how to dissect E6.5 conceptus from the uterus of pregnant mice to extract the extraembryonic ectoderm (ExE).

33. Change the medium of the MEF coated 96-well plate from MEF medium to 200 μL of TXV medium. Place the plates back in the humidified incubator (37°C with 5% CO_2) to prewarm the TXV medium.

Note: LPA and cAMP should be added to the TXV medium freshly to ensure optimal activity.

34. Sacrifice the plug positive female mice 6.5 days after plugging (day 7; 6 days after plug check).
35. Take the uteri out, and place them in PBS.

Note: At E6.5, you will clearly see the deciduae if the female has implanted concepti. As such, you do not have to proceed further if you do not find any bulb on the uterus (Figure 3A).

36. After a brief wash in PBS, remove the fat tissues with blood vessels on the mesometrium region of the uterus (Figure 3A).
37. Cut the uterus with scissors (perpendicular to the main direction of the uterus) so that each piece contains one decidua (Figures 3B–3D).
38. Place these pieces in a new 60 mm dish with PBS.
39. Insert the tip of two fine forceps into the hole formed by the uterus cutting (Figure 3D). Tear the uterine wall horizontally, and gently smooth down only the uterine wall from side to side with forceps so as to detach the decidua from the uterine wall (Figure 3E; Methods video S1).

40. Put the naked deciduae in a new 60 mm dish filled with PBS.
41. The naked deciduae have an asymmetrical oval shape with one side being larger than the other (Figure 3D). Hold the large side (blunt end) with forceps and cut off the 1/6 to 1/5 point from the small side (sharp end) with fine forceps (Figure 3F; Methods video S2).

Note: Under optimal breeding, the deciduae rarely have two concepti, and rarely does not contain a conceptus. After cutting the sharp end of the decidua, you can evaluate the number of concepti in each decidua by evaluating the amount of 'reddish debris' (Figure 3F; Methods video S2). This helps to eventually discard the deciduae that do not have a conceptus. In normal cases, you will find one clump of reddish debris. Two reddish debris on one cut means two concepti, while no reddish debris means an empty decidua.

42. Insert two tips of fine forceps very gently along the edge of the hole of the decidua which was made by decidua cutting.

⚠ CRITICAL: Since the conceptus is inside the hole (hole 2) (in Figure 3F), you should be very careful not to stab it with the tips of fine forceps.

43. Gently spread the two tips of fine forceps apart until the decidua is split in two pieces.
44. Take the transparent conceptus and transfer it into a 35 mm dish filled with 0.4% BSA (in PBS).

⚠ CRITICAL: You have to keep the ectoplacental cone (EPC) that is linked to the ExE (Figure 4A).

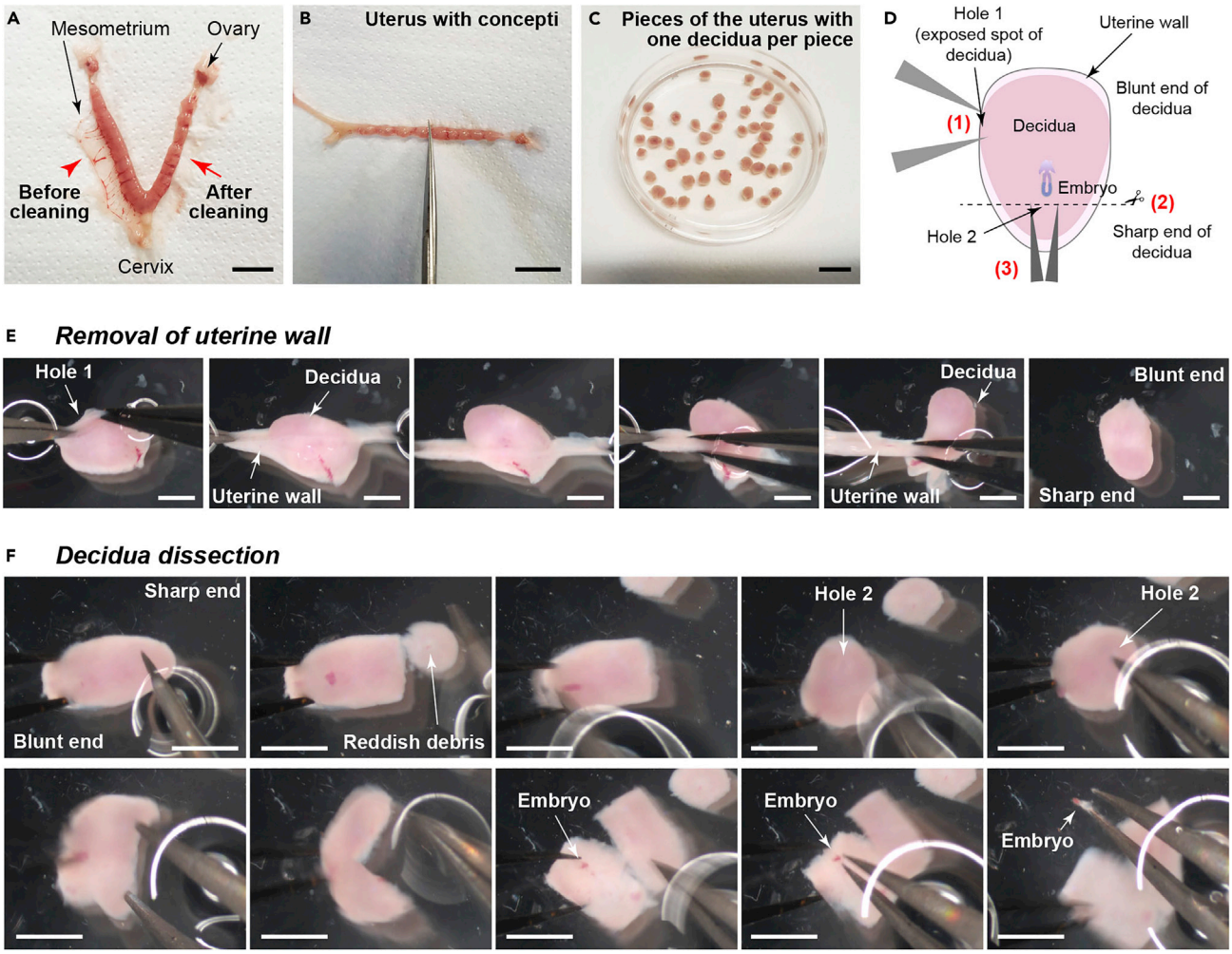

Figure 3. E6.5 Uterus and decidua dissection

(A) E6.5 uterus with implanted concepti.

(B) The uterus needs to be cut so that each piece has one decidua.

(C) Pieces of the uterus with one decidua per piece.

(D) Schematic figure explaining how to dissect E6.5 deciduae.

(E) Removal of the uterine wall. Find hole (hole 1) and stretch it using two forceps.

(F) Extraction of the E6.5 concepti from the decidua. Find a hole (hole 2) after cutting the sharp end of the decidua, and stretch it using one forceps. Scale bars: 1 cm in (A–C); 1 mm in (E and F). Related to steps 35–44. See also Methods videos S1 and S2.

⚠ CRITICAL: The Reicher's membrane is usually removed naturally when you follow the protocol. But if you find such a membrane, you should remove it. Since it is transparent and resides very close to the embryo, be careful not to damage the embryo when you remove the membrane (Figure 4B).

45. Hold the linkage of the EPC and the ExE using forceps and, using other forceps, squeeze the boundary of the epiblast (EPI) and of the extraembryonic ectoderm (ExE) to gently pull out the EPI part (Figures 4B and 4D). By pulling the EPI part away from the ExE part, one can also peel the visceral endoderm (VE) from the ExE, thus extracting a pure ExE tissue (Figure 4C; Methods video S3).

⚠ CRITICAL: The VE encloses both the ExE and the EPI. By gently squeezing the boundary of the EPI and the ExE, you can both detach the ExE from the EPI and peel the VE off.

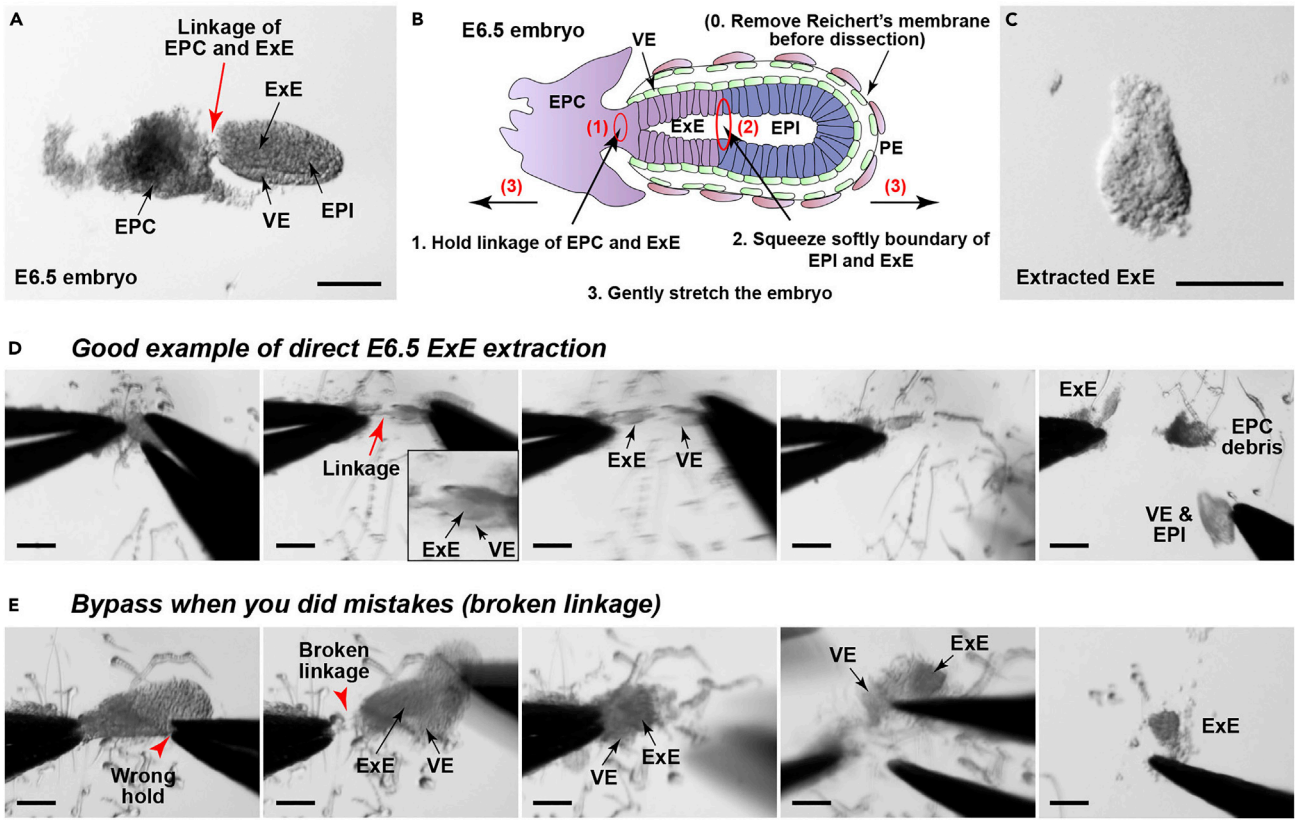

Figure 4. ExE extraction from E6.5 embryo

(A) Structure of E6.5 conceptus. If you cannot find the linkage between the EPC and the ExE, gently sweep up the EPC to reveal this linkage.

(B) Schematic figure explaining how to dissect E6.5 embryos.

(C) An example of an extracted ExE tissue.

(D) Procedures showing how to dissect an E6.5 conceptus to extract the ExE.

(E) An example of bypass when you break the linkage of the EPC and the ExE, or when you cut the EPI off from the conceptus. See problem 5 under troubleshooting. Scale bars: 100 μm in (A, C, D, and E). EPC, ectoplacental cone; ExE, extraembryonic ectoderm; EPI, epiblast; VE, visceral endoderm; PE, parietal endoderm. Related to step 45. See also Methods videos S3 and S4.

However, do not squeeze the boundary harshly so as to avoid cutting the EPI and the VE off completely (See problem 5 under troubleshooting).

⚠ CRITICAL: Since the linkage of the ExE and the EPC is fragile, you might lose this linkage when you grab the boundary of EPI and ExE improperly. For example, if you stretch the conceptus after holding only a small part of VE rather than whole EPI, you might lose the linkage because of a strong extending tension (Figure 4E; Methods video S4) (See problem 5 under troubleshooting).

Note: The ExE is distinguishable from the VE based on tissue color difference (Figures 4D and 4E).

Alternatives: You can extract ExE with chemical dissociation by using Trypsin/pancreatin.[16]

Note: By using physical dissociation, you might have more advantages as compared to chemical dissociation. Here are 4 issues during chemical dissociation. First, you might lose your samples because it becomes sticky and viscous after dissociation, making it difficult to detach the ExE from the VE and even from forceps. Second, it is difficult to distinguish the concepti in

the dissociation solutions because Trypsin/pancreatin solution on ice easily forms clumps whose color is very similar to that of the concepti. Third, incubation time on Trypsin/pancreatin is critical for the dissociation (prolonged incubation will make your samples viscous and you will lose them). If you handle many concepti, it will not be easy to timely control the dissociation process. Fourth, even though you extract the ExE using a chemical dissociation, its cells tend to have a decreased survivability in culture, as compared to the ones of physically dissociated ExE (5/13 = 38.5% from chemical dissociation, 12/13 = 92.3% from physical dissociation). In our hands, chemical dissociation also increases XEN cell contamination (6/13 = 46.2% from chemical dissociation, 0/12 = 0.0% from physical dissociation).

46. Remove the remaining EPC part from the ExE.
47. Transfer the ExE to a new 35 mm dish containing 0.4% BSA (in PBS) 1–2 times in order to wash out the debris.
48. Prepare a 20p pipette and tips, and fill them with 0.4% BSA (in PBS) to coat the inside of the tips.
49. Using the pipette and the BSA-coated 20p tips, harvest the ExE with 5 μL of 0.4% BSA (in PBS).
50. Place only one ExE in the center of one well of MEF coated 96-well plate including pre-warmed TXV medium. Incubate them in a humidified incubator at 37°C with 5% CO_2.

⚠ CRITICAL: Do not disturb the 96-well plate for at least 2 days.

Culture of cellular outgrowths and TESCs derivation

Timing: 3–5 weeks

This step describes how to culture and passage the cellular outgrowths from either blastocysts and ExEs, and TESC colonies, and how to transfer TESC colonies from MEF plates to MEF-free laminin 521-coated plates.

51. Two days after placing the blastocysts or ExEs on MEF, gently take out 100 μL out of the 200 μL of TXV medium present in each well, and add 100 μL of fresh TXV medium including a doubled concentration of the 8 factors (FGF4, Heparin, TGF-β1, Activin A, BMP7, IL-11, cAMP, LPA).

Note: You can make 2× TXV medium (without cAMP and LPA), and store it in a 4°C fridge for up to 2 weeks. However, cAMP and LPA should be added freshly to ensure optimal activity.

52. Repeat step 51 every 2 days until large cellular outgrowths have formed (see Figure 5).

⚠ CRITICAL: Avoid over-growth. Usually, cellular outgrowths need to be dissociated within 4–6 days (from E3.5 blastocysts) or 2–3 days (from E6.5 ExEs) (Figures 5A and 5B). However, each individual case should be considered every day from day 2 onward.

Note: Around 8% of samples may not form a cellular outgrowth.[1] You do not have to proceed further steps with these samples.

Summary of cell passaging

Passage	Plate preparation	Well plate	Dissociation reagent / volume	TXV medium volume	Centrifugation after dissociation
1st	0.1% gelatin / MEF	96 → 96-well	TrypLE / 35 μL	320 μL	x
2nd	0.1% gelatin / MEF	96 → 48-well	TrypLE / 35 μL	500 μL	x
3rd	Laminin 521	48 → 12-well	TrypLE / 70 μL	2 mL	o
4th (1st passage after plate conversion)	Laminin 521	12 → 6-well	Accutase / 250 μL	3–4 mL	o
After 5th (2nd passage after plate conversion)	Laminin 521	6 → 6-well	Accutase / 500 μL	3–4 mL	o

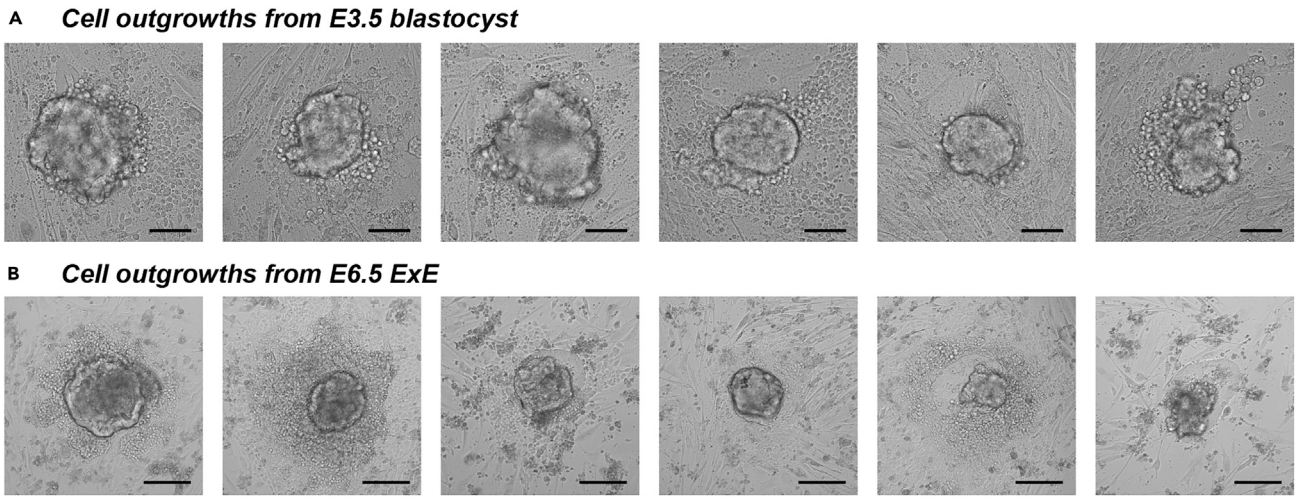

Figure 5. Various examples of cell outgrowth that are ready to be dissociated

(A and B) (A) E3.5 blastocysts cultured for 6 days and (B) E6.5 ExE cultured for 2 days. All cellular outgrowths in this figure formed TESC colonies after cell passaging. Scale bars: 100 μm in (A); 200 μm in (B). Related to step 52.

Passage 1

53. Prepare MEF in a 96-well plate (MEF medium, 20,000–25,000 cells per well) 1 day before dissociation of the cellular outgrowths.

Note: The number of MEF needs to be adjusted according to the confluency of MEF. See also steps 9–17.

54. Just before the dissociation of the cellular outgrowth, change the medium of MEF coated 96-well plate from MEF medium to 120 μL of TXV medium. Place them back into the humidified incubator to prewarm the TXV medium.

⚠ CRITICAL: 2 μM of Y-27632 (Rock inhibitor) must be added for enhanced attachment of the TESCs. No need to add Y-27632 when changing medium for already attached TESCs.

55. Remove the TXV medium from each well that contains a cellular outgrowth, and wash them with PBS.
56. Aspirate the PBS and add 35 μL of 1× TrypLE per well containing the cellular outgrowths.

⚠ CRITICAL: Use TrypLE but not Trypsin nor Trypsin-EDTA, as Trypsin does not lose its activity even after adding a large volume of TXV medium thus deteriorating or killing the cells. Using Trypsin will dramatically decrease cell viability.

57. Incubate for 8 min at 37°C with 5% CO_2.

Note: To avoid long-time exposure of the cellular outgrowth to TrypLE, do not incubate more than 8 samples at a time. Considering the cellular viability and the time-efficiency, we recommend dissociating 6–8 samples at a time.

58. After 8 min of incubation in TrypLE, gently pipet the cellular outgrowths until they dissociate into single cells. This can be monitored under conventional microscopes.
59. Add 200 μL of TXV medium onto the dissociated cells, and mix well.

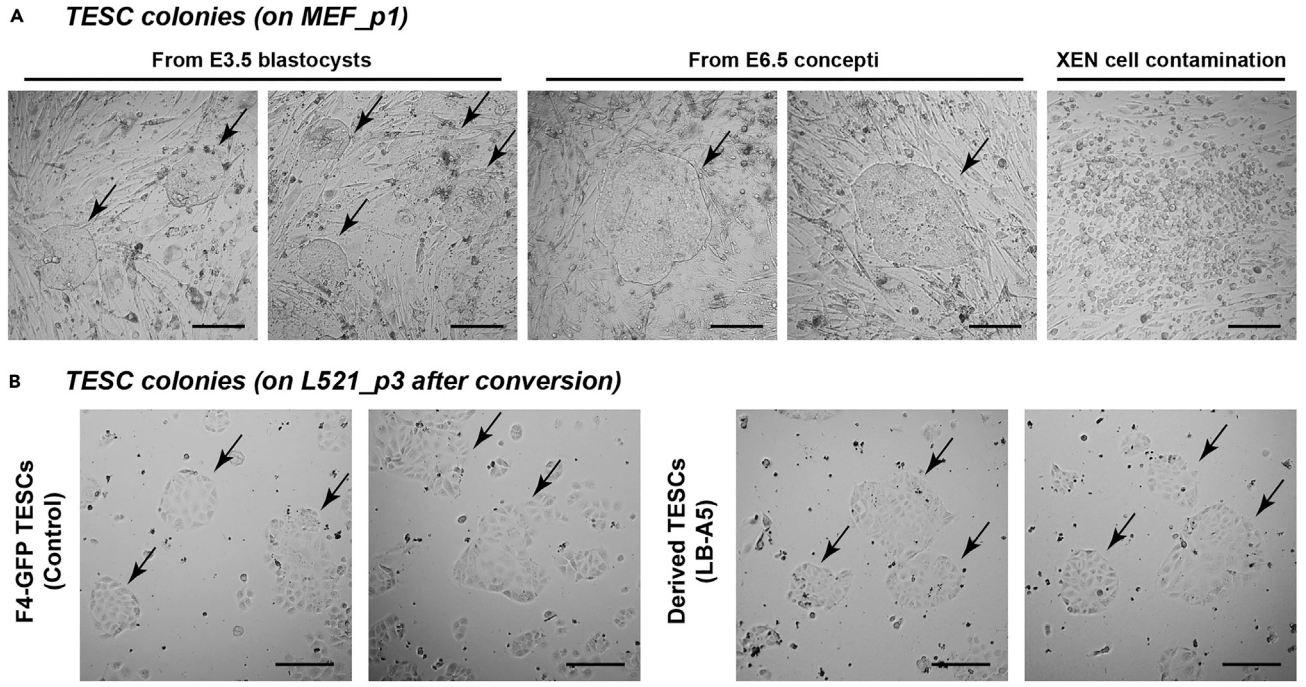

Figure 6. Various examples of TESC colonies

(A and B) (A) On MEF (p1) and (B) On Laminin 521 (p3 on MEF, and p3 after plate conversion from MEF to Laminin 521). During the early passages on MEF, TESCs form very tight and compact colonies. XEN cells are relatively smaller and more spread than TESCs, and have spherical shape as compared to TESCs. Arrows indicate TESC colonies. Scale bars: 200 μm in (A and B). Related to steps 53–113, especially steps 63 and 98.

60. Transfer the total of 235 μL of dissociated cells into the new well of MEF coated 96-well plates already including 120 μL of TXV medium per well (from step 54).
61. Incubate the plates in a humidified incubator at 37°C with 5% CO_2.

Note: Be careful when you move the plate since each well is rather filled with medium.

Note: Do not disturb the 96-well plate for at least 2 days.

62. Two days after cell dissociation, aspirate all medium and add 200 μL of fresh TXV medium.
63. Every 2 days, gently take out 100 μL of TXV medium from each well, and add 100 μL of fresh TXV medium with doubled concentration of the 8 factors until you observe large colonies of TESCs (see Figure 6).

Note: Typically, you will find small colonies around 3–4 days after cell passaging. Wait a few more days until the TESC colonies have sufficiently large sizes (Figure 6A).

Note: You might find contamination with other cell types (e.g., XEN cells) with low probability (11% for E3.5 blastocyst (5/46), and no case for E6.5 ExE (0/12)) (Figure 6A). Not every but some of these contaminated samples including other cell types will become enriched in TESCs when they are further exposed to TXV medium for a couple of additional passages.

Passage 2

64. Prepare MEF in a 48-well plate (MEF medium) at least 1 day before dissociation of TESC colonies.

Note: For a short-time culture (3–4 days) after the second passage, we did not observe that the TXV medium led to significant problems for MEF culture (e.g., detachment of MEF from plate, cell death).

65. When the size of colonies is large enough, remove the TXV medium from each well and wash them with PBS.
66. Aspirate the PBS, and add 35 μL of 1× TrypLE per well.
67. Incubate the plates for 6 min in 37°C with 5% CO_2.
68. During that time, change the medium of the MEF coated 48-well plate from MEF medium to 300 μL of TXV medium. Place these plates back into the humidified incubator to prewarm the TXV medium.
69. After 6 min of incubation in TrypLE, gently pipet the samples until they form a single cell suspension.
70. Add 200 μL of TXV medium onto the dissociated cell solution, and mix well.
71. Transfer the total volume of 235 μL into the well of the MEF coated 48-well plate that already contains 300 μL of TXV medium per well.
72. Incubate them at 37°C with 5% CO_2.

Note: From the second passage, you can observe clear TESC colonies already 2 days after cell passaging.

Passage 3

73. Prepare the MEFs in a 12-well plate (MEF medium) at least 1 day before dissociation of the TESC colonies.
74. When the size of the colonies is large enough, remove the TXV medium from each well and wash them with PBS.
75. Aspirate the PBS, and add 70–80 μL of 1× TrypLE per well.
76. Incubate the plates for 6 min in 37°C with 5% CO_2.
77. In the meantime, prepare 1.0–1.5 mL of washing buffer in 1.5–2.0 mL tubes.
78. Change the medium of the MEF-coated 12-well plate from MEF medium to 1 mL of TXV medium. Place them back into the humidified incubator to prewarm the TXV medium.
79. After 6 min of incubation in TrypLE, gently pipet the samples until they form a single cell suspension.
80. Transfer the dissociated cell suspension into the 1.5 mL tubes containing a washing buffer.
81. Centrifuge the tubes for 4 min at 200 g.
82. Remove the supernatant and tap the pellet several times with the remaining washing buffer in order to roughly dissociate it.
83. Add 1 mL of TXV medium onto the pellet and resuspend the cells.
84. Transfer a total volume of 1 mL of dissociated cells into the MEF-coated 12-well plate containing 1 mL of TXV medium per well.
85. Incubate the plates in 37°C with 5% CO_2.

⚠ CRITICAL: From the third passage, do not simply dilute TrypLE with a large volume of TXV medium as it can decrease cell viability. Instead, use a wash buffer and centrifuge them to remove remaining TrypLE and resuspend the cells in pure TXV medium.

Passage 4 (passage 1 after plate conversion)

86. Prepare the MEF in a 12-well plate (MEF medium) at least 1 day before dissociation of the TESC colonies.
87. Prepare the Laminin 521 (L521) coatings in a different 12-well plate at least 4 h before the dissociation of TESC colonies. Keep the plate at 37°C with 5% CO_2 incubator.

Note: You can keep the L521-coated plates in the fridge for up to 2 weeks by sealing them with parafilm to prevent the evaporation of the solution. To properly coat the plates with L521, it is required to keep the L521 solution in the plate and in the fridge at least for 1 day. In such a case, you do not have to incubate the plate at 37°C with 5% CO_2 incubator before cell passaging.

Note: You need 500–700 μL of L521 to coat one well of a 12-well plate.

88. When the size of the colonies is large enough, remove the TXV medium from each well and wash them with PBS.
89. Aspirate the PBS and add 250 μL of Accutase per well.

Note: Accutase is milder than TrypLE and is preferred upon passaging to laminin coated plates. To minimize cell damage by dissociation reagents, we strongly recommend use of Accutase instead of TrypLE or Trypsin-EDTA.

90. Incubate the plates for 6 min at 37°C with 5% CO_2.
91. In the meantime, prepare 3–4 mL of washing buffer in 15 mL tubes.
92. Change the solution of the laminin-coated 12-well plates to 1.2 mL of TXV medium. Place them back into the humidified incubator to prewarm the TXV medium.
93. After 6 min of incubation in Accutase, gently pipet the samples until they form a single cell suspension.
94. Transfer the dissociated cell solution to 15 mL tubes including a washing buffer.
95. Centrifuge the tubes for 4 min at 200 g.
96. Remove the supernatant and tap the pellet several times with the remaining washing buffer to roughly dissociate it.
97. Add 1 mL of the TXV medium into the tube and resuspend the pellet.
98. Take 800 μL of resuspended TESCs and transfer them into the laminin-coated 12-well plate which contains 1.2 mL of TXV medium per well.

⚠ CRITICAL: Before the third passage, plate conversion to the L521 plate is not successful in our hands. We typically try this conversion after the third passage (Figure 6B).

Note: When attempting plate conversion from MEF- to L521-coated plates, 3–10 times more cells should be seeded as compared to normal cell passaging because many TESCs will not firmly attach to the plate.

Note: We highly recommend seeding remaining TESCs on MEF-coated plates as a backup in case the conversion to laminin culture fails.

99. Incubate the plates at 37°C with 5% CO_2.

After passage 5 (passage 2 after plate conversion)

100. Prepare Laminin 521 (L521) coating in one well of a 6-well plate or on a 35 mm culture dish at least 4 h before (in 37°C with 5% CO_2) or 1 day before (in the fridge) cell passaging.

Note: You need 1.0–1.3 mL of L521 to coat one well of a 6-well plate.

101. Remove the TXV medium from each well and wash them with PBS.
102. Aspirate the PBS, and add 500 μL of Accutase per well.
103. Incubate them for 6 min in 37°C with 5% CO_2.
104. In the meantime, prepare 5–6 mL of washing buffer in 15 mL tubes.

105. After 6 min of incubation in Accutase, gently pipet the samples until they form a single suspension.
106. Transfer the dissociated cells into 15 mL tubes containing washing buffer.
107. Centrifuge the tubes for 4 min at 200 g.
108. Remove the supernatant and tap the pellet several times with the remaining washing buffer to roughly dissociate it.
109. Add 400–500 μL of TXV medium and resuspend the TESCs.
110. Count the number of cells.
111. Seed 7.0×10^4–1.0×10^5 of TESCs per one well of a 6-well plate with 3–4 mL of TXV medium + 2 μM of Y-27632.
112. Change the medium 2 days after passaging.
113. Passage the TESCs again 3 days after the last passage by repeating steps 101–113.

Immunofluorescence

Timing: 2 days

This step describes how to confirm that the newly derived cell lines are genuine TESCs by immunofluorescence (IF) of 2D cells and by blastoid formation. This staining procedure is applied to both ESCs and TESCs.

Note: TESC Derivation (steps 51–113) → IF of 2D TESCs (steps 114–124) → making blastoid with the TESCs (steps 125–143) → IF of the blastoid (steps 114–124).

114. Remove the TXV medium from each well and wash them with PBS.

Note: Check if your microscope and your culture dishes are compatible for imaging.

Alternatives: You can use specific dishes for high quality imaging (e.g., glass bottom plate, ibiTreat plastic plate).

115. Aspirate the PBS, and add 1 mL of 4% Formaldehyde solution (FA. Or add paraformaldehyde, PFA) for one well of a 6-well plate. Incubate them for 30 min at 20°C–25°C.
116. Wash them with PBS (5 min × at least 3 times).
117. For permeabilization, remove the PBS and add 1 mL of 0.3% PBS-T. Incubate them for 30 min in 20°C–25°C.
118. Remove the 0.3% PBS-T solution and add a blocking buffer. Incubate them for at least 1 h at 20°C–25°C.

Note: When making the blocking buffer, it is recommended to use serum of the same species as the secondary antibody host. For example, if you want to use anti-rabbit goat 488 and anti-rabbit donkey 488 secondary antibodies, use goat and donkey serum for blocking buffers, respectively.

Note: If you want to pause after this step, you can keep the samples at 4°C overnight.

119. Prepare a primary antibody solution with the blocking buffer. Incubate the samples with the primary antibody solution at 4°C overnight.

Note: We used CDX2/EOMES and NANOG/OCT4 for the trophoblast and epiblast markers, respectively. One can also use GATA3 as a trophoblast marker. As controls, we also prepared well-established ESC and TSC lines (e.g., V6.5 ESCs and F4-GFP TSCs) (See also Figure 7A).

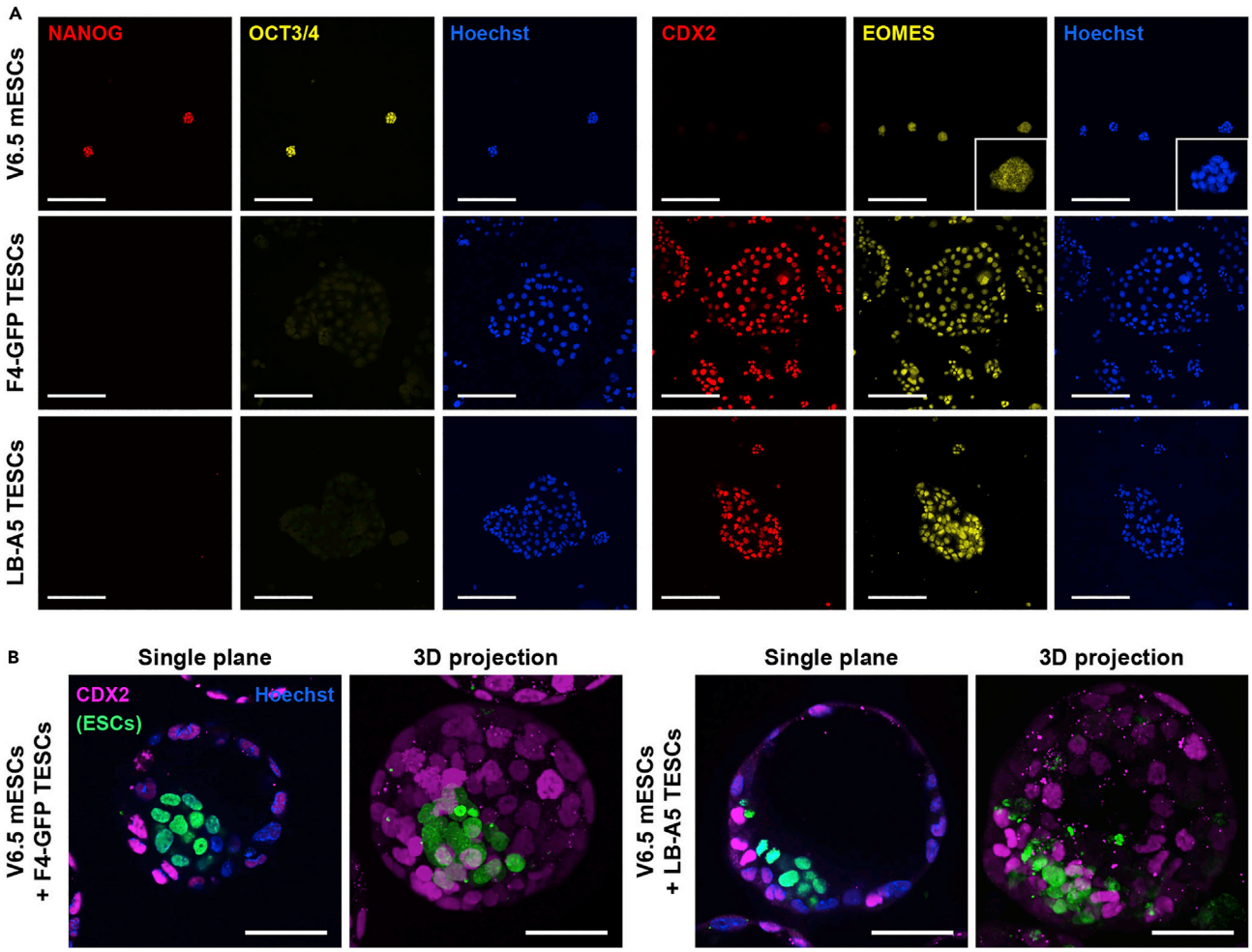

Figure 7. Immunofluorescence of 2D TESCs and 3D blastoids made with new derived (LB-A5) TESCs

(A) Immunofluorescence for epiblast and trophoblast lineage markers in ESCs and TSCs. EOMES was also detected in ESCs, with a cytoplasmic but not nuclear localization, suggesting a non-specific background signal. On the contrary, EOMES in TESCs is clearly detected in the nucleus of cells.

(B) Blastoids generated with ESCs-H2B-mTurquoise and either F4-GFP or LB-A5 TESCs, and immunofluorescence for CDX2. Both blastoids established a polar-mural CDX2 axis, like late blastocysts. ESCs were identified by naive mTurquoise signals. Scale bars: 200 μm in (A); 50 μm in (B). Related to steps 115–124.

Alternatives: You can incubate your samples with the primary antibody solution at 20°C–25°C for 2 h. However, the intensity of fluorescence might decrease, while the background signal might increase.

120. Wash the samples with 0.1% PBS-T (5 min × at least 3 times).
121. Prepare a secondary antibody solution by adding the secondary antibodies and Hoechst in the blocking buffer. Incubate the samples with the secondary antibody solution for 1 h at 20°C–25°C.
122. Wash them with 0.1% PBS-T (5 min × at least 3 times).
123. Wash out the remaining 0.1% PBS-T with PBS, and image your samples (Figure 7A).
124. Once you have confirmed that the newly derived cell lines express trophoblast lineage markers but not epiblast markers, you can engage in making blastoids[11,17] to assess the functional potential of cells to recapitulate aspects of trophectoderm development (Figure 7B).

Note: We routinely use multiple lines of V6.5 ESCs (in 2i-Lif medium on 0.1% gelatin-coated plate) and F4-GFP or new derived TESCs (in TXV on L521 coated plate) to form blastoids.

Note: If the new derived cell lines are not genuine TESCs, they don't form a blastocoel-like cavity, but rather compact cellular aggregates.

Blastoid formation

Timing: 4 days

This step describes how to make blastoids using ESCs and TESCs.

125. Prepare 200 μm diameter non-adherent hydrogel microwells (96- or 12-well plates).[18]

Note: You can keep microwells in fridge up to 2 months by adding PBS on them.

126. At least 4 h before your experiment, remove PBS from the microwells and add mES medium (150 μL for 96-well; 1 mL for 12-well plate) and incubate them at 37°C with 5% CO_2 for the equilibrium of medium in microwells.

Alternatives: You can change PBS to mES medium 1 day before experiment. Keep them in an incubator (37°C with 5% CO_2) until you use them.

127. After wash with PBS, detach ESCs using Accutase. Incubate them for 4 min at 37°C with 5% CO_2.
128. Centrifugate detached ESCs according to steps 104–108.
129. Resuspend ESCs in mES medium, and count the number of ESCs.

Note: If you want to maintain ESC line for a next experiment, resuspend ESCs in basal B27N2 medium but not in mES medium to avoid exposure to serum.

130. Remove mES medium from the microwells and add fresh mES medium to prevent drying of the microwells (25 μL for a 96-well; 150 μL for a 12-well).
131. Seed 8–10 cells per microwell (total volume of mES medium would be 50 μL for a well of a 96-well and 150 μL for a 12-well plate).
132. Incubate ESCs at 37°C with 5% CO_2 for 20 min to settle them down on microwells.
133. Add blastoid mES medium (150 μL for a 96-well and 1 mL for a 12-well plate) and incubate them at 37°C with 5% CO_2 for 26–28 h.
134. Check if they make sphere shaped aggregate.

Note: If ESCs did not form proper aggregate, check if your microwells are contaminated or too old.

135. After wash with PBS, detach TESCs using Accutase. Incubate them for 6 min at 37°C with 5% CO_2.
136. Centrifugate detached TESCs according to steps 104–108.
137. Resuspend TESCs in basal TX medium, and count the number of TESCs.
138. Remove mES medium from the microwells gently and add fresh basal TX medium to prevent drying of the ESC aggregates (25 μL for a 96-well; 150 μL for a 12-well).

⚠ CRITICAL: Aspirate mES medium gently and add basal TX medium slowly not to disturb ESC aggregates. Harsh flow of medium might cause ESC aggregates to float.

139. Seed 16–18 cells per microwell (total volume of medium would be 50 μL for a well of a 96-well and 150 μL for a 12-well plate).

140. Incubate ESCs at 37°C with 5% CO_2 for 20 min to settle them down on microwells.
141. Add blastoid TX medium (150 μL for a 96-well and 1 mL for a 12-well plate) and incubate them at 37°C with 5% CO_2.
142. 24 h after TESC seeding, add 1.5 mM of cAMP per microwell.

Note: If the volume of 1.5 mM of cAMP is less than 10 μL, they might not be spread well. So, put 1.5 mM of cAMP into basal TX medium to make total 10 μL of solution. Add the solution on microwell.

143. Harvest blastoids 65–68 h after TESC seeding (41–44 h after cAMP shot).

Note: Harvest only cavitated and sphere-shaped blastoids.

EXPECTED OUTCOMES

Obtaining high-quality cell lines reflecting a specific, restricted developmental stage is crucial to forming relevant embryo models predictive of development. So far, TS (trophoblast stem cell medium with serum) and TX (trophoblast stem cell medium without serum) medium have been mainly used for TSC derivation and culture. However, TSC represented a mix of pre-implantation and post-implantation stages. TESCs derived and cultured in TXV medium better reflect the trophectoderm of blastocyst and such cell lines are useful as a model of pre-implantation stage. The needs of specific TESC lines may vary depending on research interests that can be largely divided into 1) the genetic modification to be performed, and 2) the mouse strain background necessary for a specific experiment. Genetically modified TESCs can be obtained by transfecting vectors, however, in some cases, direct derivation from genetically-modified mice is preferred, for example when a complex genetic modification is needed from a specific, well-characterized mouse colony. TXV medium also allows for the derivation of TESC lines with high efficiency (from E3.5 and E6.5, 95.7% and 83.3% of efficiency, respectively) as compared to more sub-optimal TS and TX medium (0–50% depending on mouse strain),[4,7] and regardless of the origin of the mouse strain that we tested (in 129/Sv, FVB/N, C57BL/6J and B6CBAF1).

After deriving TESC lines, their quality must be tested by first evaluating the cell growth speed, morphology, and potentially, in rare cases, their contamination with other cell types. Based on these basic criteria, several TESC lines can be selected to form blastoids as a good criterion for further assess their quality. We tested our newly derived TESC lines and found that most of them formed morphologically good blastoids. In addition, the embryo dissection method described here is not only used for TESC derivation but may help many young scientists who want to study early embryology.

LIMITATIONS

Here, the efficiency of derivation of lines of TECSs and of blastoid formation was especially examined using 129/Sv, FVB/N, C57BL/6J and B6CBAF1 mouse strains. Additional stringent tests using other mouse strains are required.

During E6.5 conceptus dissection, we intended to increase cell viability and derivation efficiency by reducing the usage of chemicals (e.g., trypsin and pancreatin). However, because surgical dissections are performed manually, it is necessary to practice sufficiently before dissecting important samples.

TROUBLESHOOTING

Problem 1

Even after superovulation, plugging or pregnancy efficiency is very low (step 7).

Potential solution

Avoid using 6–10 week-old female mice for superovulation since injected hormones tend to not work properly because of high level of endogenous sex hormones. It will decrease plugging efficiency. For some mouse strains which develop and grow fast (e.g., FVB/N, ICR), females aged 4–5 weeks are also suitable for hormone priming.

Problem 2

MEF keeps dying and becomes bad after putting blastocyst on the MEF (step 17).

Potential solution

MEF might deteriorate due to the long-term exposure to TXV medium (serum-free medium). We recommend preparing MEF as late as possible (just 1 day before use), starting with a density of 20,000–25,000 cells per well (for one well of a 96-well plate), and adjusting the confluency of your feeder cells. The MEF should cover the entire plate and be confluent. Adding additional MEFs during the culture of the cellular outgrowth did not work on our hands, so please make sure that the number of MEFs is sufficient before starting your experiment.

Problem 3

Obtaining only a few blastocysts after uterus flushing (step 25).

Potential solution

The first critical step for uterus flushing is the inserting of a needle. Make sure that the tip of the needle does not penetrate through the opposite uterine walls but stays in the uterus cavity by inserting the needle in a direction parallel to the uterus length. You do not have to insert a needle deeply, but you need to hold the uterus and needle tightly together with forceps. Swollen uterus during flushing indicates that you are flushing the uterus properly.

Problem 4

There are only a few blastocysts after uterus flushing (step 29).

Potential solution

You will find around 10–30 blastocysts from one mouse (two horns). If you obtain only a few blastocysts from one mouse (e.g., 1–4 blastocysts), it might mean that the flushing process is not appropriate. If the initial flushing is inefficient, we recommend you flush again by inserting the syringe needle into the cervix side of the uterus horn (in the opposite direction to the first flushing). You might not flush the blastocysts because of a deeply inserted needle (e.g., if the length of the uterus is 2 cm, and if you insert your needle 1 cm deep, you will not be able to flush half of the blastocysts). If you cannot find any blastocyst even after re-flushing, it is likely that the mouse was not pregnant.

Problem 5

The linkage of the EPC and the ExE is broken during dissection. Or the boundary of the EPI and the ExE is cut by mistake (step 45).

Potential solution

1) When you lose the linkage of the ExE and the EPC, cut the EPI off from the conceptus. Hold the VE near the linkage (left hand), and scoop the ExE out from the VE (right hand) by gently squeezing the VE (left hand). 2) When you cut the boundary of the EPI and the ExE, 2-1) remove the EPC and repeat 1). Or 2-2) hold the linkage of the ExE and the EPC or the VE near the linkage (left hand), and insert two tips of forceps into the boundary of the ExE and the VE (right hand). Gently spread apart two tips to tear the VE, and scoop the ExE out from the VE (see also Methods video S4).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Nicolas C. Rivron (nicolas.rivron@imba.oeaw.ac.at).

Materials availability

The study did not generate new unique reagents.

Data and code availability

No data or code was generated or analyzed in this protocol.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102151.

ACKNOWLEDGMENTS

J.S. is supported by the European Union's Framework Programme for Research and Innovation Horizon 2020 (2014–2020) under the Marie Curie Skłodowska Grant Agreement Nr. 847548. N.C.R. is supported by an ERC Consolidator grant (2020 ERC-CoG No:101002317-BLASTOID).

AUTHOR CONTRIBUTIONS

Conceptualization, J.S., N.C.R.; Formal analysis, J.S.; Funding acquisition, J.S., N.C.R.; Investigation, J.S.; Methodology, J.S., N.C.R.; Validation, J.S., N.C.R.; Visualization, J.S.; Writing – original draft, J.S., N.C.R.; Writing – review & editing, J.S., N.C.R.; Project administration, N.C.R.; Supervision, N.C.R.

DECLARATION OF INTERESTS

N.C.R. is an inventor on the patent "Blastoid, cell line based artificial blastocyst" (EP2986711) and on the patent application "Blastocyst-like cell aggregate and methods" (EP21151455.9), both maintained by the Institute for Molecular Biotechnology, Austrian Academy of Science.

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