Protocol

Derivation and Culture of Epiblast Stem Cell (EpiSC) Lines

Peter Rugg-Gunn

This protocol describes the derivation and culture of epiblast stem cells (EpiSCs) from early postimplantation epiblasts. EpiSCs can be maintained in an undifferentiated state and retain the ability to generate tissues from all three germ layers in vitro and to form teratomas in vivo. However, they seem unable to form chimeras. Whether this is due to differences in developmental status or a cellular incompatibility (e.g., cell adhesion) between EpiSCs and the host inner cell mass (ICM) is currently unclear. Other differences between mouse embryonic stem (ES) cells and EpiSCs also exist, including gene expression profiles and different growth factor requirements for self-renewal. Thus, EpiSCs provide an important in vitro model for studying the establishment and maintenance of pluripotency in postimplantation epiblast tissues.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Activin A stock solution (10 µg/mL) (ActA; R&D Systems 338-AC-005) <R>

BSA fraction V solution in PBS (0.1%, w/v) <R>

CDM + FGF2 + ActA medium <R>

Cell dissociation buffer (Invitrogen 13150-016)

Supplement with 3 mg/mL BSA to prevent embryo sticking. Filter-sterilize.

Chemically defined medium (CDM) <R>

Collagenase type IV (1 mg/mL) <R>

Early postimplantation embryos (5.5–6.5 dpc [days postcoitum])

Embryo-handling medium (e.g., FHM, Millipore MR-024)

EpiSC medium <R>

EpiSC + FGF2 medium <R>

FGF2 stock solution (10 µg/mL) (R&D Systems 233-FB) <R>

Fibronectin (10 mg/mL) (Chemicon FC010)

Flushing and handling medium (FHM), Ca²⁺/Mg²⁺ free

Obtain commercially by special order from embryo culture media supply company.

From the Manipulating the Mouse Embryo collection, edited by Richard Behringer, Marina Gertsenstein, Kristina Vintersten Nagy, and Andras Nagy.

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Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot093971

Freezing medium for feeder cell culture (2×) <R>
Freezing medium for feeder-free culture (1×) <R>
Gelatin (0.1%, w/v, in water, autoclaved) (Swine Skin type II, Sigma-Aldrich G2500)
MEF feeder layer plates (see Protocol: **Preparing Mouse Embryo Fibroblasts** [Nagy et al. 2006a],
Protocol: **Preparing Feeder Cell Layers from STO or Mouse Embryo Fibroblast (MEF) Cells:
Treatment with Mitomycin C** [Nagy et al. 2006b], and Protocol: **Preparing Feeder Cell Layers from
STO or Mouse Embryo Fibroblast (MEF) Cells: Treatment with γ -Irradiation** [Nagy et al. 2006c])
Pancreatin/trypsin solution <R>
Phosphate-buffered saline (PBS) <R>
Prepare PBS without added Ca^{2+} and Mg^{2+} . Supplement with 3 mg/mL BSA to prevent embryo sticking, and then filter-sterilize.

Equipment

Biosafety cabinet
Embryo-handling pipette (e.g., a finely drawn Pasteur pipette, i.d. = $\sim 100\ \mu\text{m}$, attached to an aspirator mouthpiece)
Forceps
Glass scalpels
Fuse the central portion of a glass capillary (o.d. of 1 mm, i.d. of 0.75 mm) over a flame, and generate a tapered cutting surface using an electrode puller. For more details, see Downs (2006).
Incubator, humidified, 37°C; 5% CO_2 , 95% air
Microscopes, inverted and stereomicroscope
Needles (30 gauge)
Non-tissue-culture-treated dishes (e.g., 35 mm)
Pipettes
Pipettors, automatic (P20 and P200)
Tissue-culture dishes (various sizes, including 24-well plates)

METHOD

Derivation of EpiSC Lines

1. Derive the EpiSC cell lines from early postimplantation embryos, using either feeder cells or feeder-free conditions.

For Derivation on MEF Feeders

- i. The day before isolating the embryos, prepare gelatin-coated 24-well tissue-culture plates with MEFs at 4×10^4 cells/cm².

*MEFs derived from 13.5-dpc embryos work best (see Protocol: **Preparing Mouse Embryo Fibroblasts** [Nagy et al. 2006a], Protocol: **Preparing Feeder Cell Layers from STO or Mouse Embryo Fibroblast (MEF) Cells: Treatment with Mitomycin C** [Nagy et al. 2006b], and Protocol: **Preparing Feeder Cell Layers from STO or Mouse Embryo Fibroblast (MEF) Cells: Treatment with γ -Irradiation** [Nagy et al. 2006c]).*

Days 1–2

- ii. In the morning of isolating the embryos, replace the medium with EpiSC + FGF2 medium (500 μL /well).
- iii. Collect 5.5–6.5 dpc embryos under sterile conditions in FHM, and remove Reichert's membrane using sharp forceps or 30-gauge needles.

- iv. Using a 30-gauge needle or glass scalpel, cut the embryo near the boundary of the embryonic and extra-embryonic regions. Transfer the embryonic fragment (with overlying endoderm) to a drop of ice-cold pancreatin/trypsin and incubate for 5–8 min at 4°C. Return the fragment to FHM and allow it to rest for 5 min.
- v. Using the finely drawn glass pipette, carefully draw the embryonic fragment into the pipette (cut end first). The epiblast should peel away from the visceral endoderm and enter the pipette.
- vi. Transfer each epiblast to an individual well of the 24-well plate containing EpiSC + FGF2 medium and culture for 24 h at 37°C and 5% CO₂ (Fig. 1A).

Days 3–4

- vii. Change the medium after 48 h.
The epiblast outgrowth is ready for passage on Days 3–4 (see Fig. 1B).
- viii. Using a glass pipette or 30-gauge needle, cut the colony into four to six equally sized pieces, and transfer the pieces to a fresh well of MEF feeders. Leave behind the extra-embryonic cells that are present in the colony because of poor initial isolation (Fig. 1C,D).

For Derivation in Feeder-Free Conditions

- i. The day before isolating the embryos, coat 24-well culture dishes with fibronectin. To each well, add 500 µL of PBS and 5 µL of fibronectin, swirl to coat, and incubate at 37°C. Use the plate after 1 h and within 24 h. Just before use, remove the solution, wash once with PBS, and add CDM + FGF2 + ActA.

FIGURE 1. Derivation of epiblast stem cells on MEFs. (A) Typical epiblast colony after 2 d of culture on fibroblast feeders. (B) The same colony after one additional day of culture. (C,D) Examples of epiblast colonies where extra-embryonic tissues were not completely removed before plating (indicated by arrows). (Reprinted with permission from Chenoweth and Tesar 2010, © Springer Science + Business Media.)

Day 1

- ii. Replace the fibronectin with CDM + FGF2 + ActA (500 μ L/well) in the morning of isolating the embryos.
- iii. Collect 5.5–6.5 dpc embryos under sterile conditions in FHM, and remove Reichert's membrane using sharp forceps or 30-gauge needles.
- iv. Wash the embryos in PBS, Ca^{2+} / Mg^{2+} -free, containing 3 mg/mL BSA, and incubate in cell dissociation buffer for 15–20 min at 4°C. Transfer the embryos to a 35-mm dish containing ice-cold FHM, Ca^{2+} / Mg^{2+} -free.
- v. While holding the embryo down on the dish with one needle, make small tears near the boundary of the embryonic and extra-embryonic regions using the tip of another needle. As the tear opens up, the visceral endoderm will become easier to reflect and dissect off the epiblast (the endoderm usually comes off as a single sheet of cells). Sever the epiblast from the extra-embryonic ectoderm.
- vi. Transfer each epiblast to an individual well of the 24-well culture dish containing CDM + FGF2 + ActA, and incubate at 37°C and 5% CO_2 .

Days 2–5

- vii. Change the medium after 24 h and repeat daily.

The EpiSC colonies should be large and ready for passage by Days 6–10.

Days 6–10

- viii. Add collagenase type IV (0.5 mL/well of a 24-well plate) and incubate for 1–2 min at room temperature. Carefully remove collagenase and add 0.5 mL of CDM. Using a P20 pipette, pick EpiSC colonies and transfer cell clumps to a new well containing CDM + FGF2 + ActA.
2. From now on, change the medium daily (using CDM + FGF2 + ActA for cells grown with feeder cells or using EpiSC + FGF2 for feeder-free conditions). Passage the cells using collagenase type IV. Distribute the cells at a 1:1 or 1:2 ratio for the first few passages.

Culturing EpiSC Cells

3. Culture EpiSCs with or without MEF feeder cells.

To Culture Cells on MEF Feeder Cells

- i. Prepare gelatin-coated tissue-culture dishes with MEF feeders at a density of 4×10^4 cells/ cm^2 . MEFs derived from 13.5-dpc embryos work best (see Protocol: **Preparing Mouse Embryo Fibroblasts** [Nagy et al. 2006a], Protocol: **Preparing Feeder Cell Layers from STO or Mouse Embryo Fibroblast (MEF) Cells: Treatment with Mitomycin C** [Nagy et al. 2006b], and Protocol: **Preparing Feeder Cell Layers from STO or Mouse Embryo Fibroblast (MEF) Cells: Treatment with γ -Irradiation** [Nagy et al. 2006c]).
- ii. Culture EpiSCs on MEF feeders in EpiSC + FGF2 medium in a standard tissue-culture incubator (37°C, 5% CO_2). Change the medium every day and passage the cells (1:4–1:6) every 2–3 d or when the culture has reached $\sim 50\%$ confluency. The optimum density of EpiSCs is variable and should be determined for each line.
- iii. Add collagenase type IV (e.g., 1 mL/well of a six-well plate) and incubate for 8–12 min at 37°C. Add 1 mL of EpiSC medium to each well, gently dislodge the colonies by pipetting with a 5-mL pipette, and transfer the cells to a 15-mL conical tube. Collect the colonies by centrifugation at 200g for 15 sec.

- iv. Remove the supernatant, and gently resuspend the colony pellet in 5 mL of EpiSC medium. Collect the colonies by centrifugation at 200g for 15 sec, and repeat the wash and centrifugation once more (for a total of three times).
- v. Resuspend the colonies in 100 μ L of EpiSC medium, and transfer them to one well of a 96-well round-bottom plate. Gently break up the colonies into small clumps using a P200 pipette. Transfer the clumps onto fresh MEF feeders in EpiSC + FGF2 medium.

To Culture Cells in Feeder-Free Conditions

- i. Prepare the plates by coating using one of the following options:
 - *For treatment with fibronectin.* Add 500 μ L of PBS and 5 μ L of fibronectin to each well of a 12-well plate. Swirl to coat and incubate at 37°C. Use the plate after 1 h and within 24 h. Just before use, remove the solution, wash once with PBS, and add CDM + FGF2 + ActA.
 - *For treatment with gelatin followed by serum.* Coat each well with gelatin and replace it with MEF media (containing at least 10% fetal bovine serum [FBS]) (Protocol: **Preparing Mouse Embryo Fibroblasts** [Nagy et al. 2006a]). Incubate overnight at 37°C and use within 1 wk. Just before use, remove the media, wash once with PBS, and add CDM + FGF2 + ActA.
- ii. Culture the EpiSCs on fibronectin- or serum-coated dishes in CDM + FGF2 + ActA medium in a standard tissue-culture incubator (37°C, 5% CO₂). Change the medium every day and passage the cells (1:4–1:6) every 2–3 d or when the culture has reached ~50% confluency. The optimum density of EpiSCs is variable and should be determined for each line.
- iii. Add collagenase type IV (e.g., 0.5 mL/well of a 12-well plate) and incubate for 5–8 min at room temperature. Gently dislodge the cells from the plate using the tip of a 5-mL pipette or cell scraper, and transfer the cells to a 15-mL conical tube.
- iv. Add 5 mL of CDM, collect the cells by centrifugation at 200g for 3 min, and wash once more with CDM. The cells should not need much trituration—the ideal size clump should contain ~500–1000 cells (~300 μ m).
- v. Resuspend the cells in CDM + FGF2 + ActA and transfer to fresh fibronectin-coated or serum-coated dishes.

Cryopreservation of EpiSCs

4. Freeze and thaw the EpiSCs using procedures similar to those described for ES cells (Protocol: **Freezing and Thawing of Embryonic Stem (ES) Cells Using Cryovials** [Nagy et al. 2006d]). Prepare freshly prepared freezing medium and keep on ice before adding to the cells.
 - i. *To freeze EpiSCs cultured on MEFs.* Add 2 $\times$ freezing medium for feeder cell culture to the cell suspension in equal volume.
 - ii. *To freeze EpiSCs cultured in feeder-free conditions.* Add 1 $\times$ freezing medium directly to the cell pellet obtained from the feeder-free culture.

DISCUSSION

Although the isolation of epiblast tissue from the early postimplantation embryo requires careful dissection, once the tissue has been obtained, the efficiency of establishing an EpiSC line is very high (close to 100%). The procedures above are based on publications by Brons et al. (2007), Tesar et al. (2007), Guo et al. (2009), and Greber et al. (2010). For more details, see Chenoweth and Tesar (2010).

Alternatively, stable EpiSC lines may also be derived from the preimplantation embryo (Najm et al. 2011). Although the derivation efficiency appears to be lower (reported to be 26%), this

approach may be technically easier for many laboratories, and you are referred to Najm et al. (2011) for further details. Irrespective of their method of derivation, EpiSCs require FGF2 and Activin A to maintain an undifferentiated state and can be propagated on MEF feeders or in feeder-free conditions.

Stable EpiSC-like cell lines can also be obtained from in vitro differentiation of ES cells. The protocol involves transferring ES cells into EpiSC culture conditions (e.g., Activin and FGF supplementation) and propagating the resulting EpiSC-like colonies. For more details, see Guo et al. (2009), Greber et al. (2010), and Zhang et al. (2010).

RECIPES

Activin A Stock Solution (10 µg/mL)

Reagent	Amount
Recombinant Activin A (ActA; R&D Systems 338-AC-005)	5 µg
BSA fraction V solution in PBS (0.1%, w/v) <R>	0.5 mL
Resuspend lyophilized Activin A in its vial, mix well, aliquot 50 µL, and freeze at −80°C. Thaw each aliquot as needed and store it at 4°C. Do not refreeze.	

BSA Fraction V Solution in PBS (0.1%, w/v)

Reagent	Amount
BSA fraction V (Sigma-Aldrich A3311 or A1470)	10 mg
Phosphate-buffered saline (PBS) without Ca ²⁺ /Mg ²⁺ <R>	10 mL
Resuspend BSA in PBS. Filter through a 0.45-µm filter, aliquot, and store at −80°C.	

Ca²⁺/Mg²⁺-Free Tyrode Ringer's Saline

NaCl, 8.0 g
 KCl, 0.3 g
 NaH₂PO₄·5H₂O, 0.093 g
 KH₂PO₄, 0.025 g
 NaHCO₃, 1.0 g
 Glucose, 2.0 g
 Dissolve in 1 liter of H₂O. Adjust pH to 7.6–7.7.

CDM + FGF2 + ActA Medium

Reagent	Stock concentration	Final concentration	Amount
FGF2 stock solution <R>	10 µg/mL	12 ng/mL	12 µL
Activin A stock solution <R>	10 µg/mL	20 ng/mL	20 µL
Chemically defined medium (CDM) <R>			10 mL
Store at 4°C and use within 1 wk.			

Chemically Defined Medium (CDM)

Reagent	Amount	Final concentration
F-12 Nutrient Mixture (Ham) with GlutaMAX (Invitrogen 31765-035)	250 mL	
Iscove's modified Dulbecco's medium (IMDM) with GlutaMAX (Invitrogen 31980-030)	250 mL	
BSA ^a fraction V (e.g., Equitech-Bio, Inc., BAC62 or Sigma-Aldrich A1470)	2.5 g	5 mg/mL
Thioglycerol (11.5 M stock; Sigma-Aldrich M7522)	20 μ L	450 μ M
Insulin (10 mg/mL stock; Roche 11376497001)	350 μ L	7 μ g/mL
Transferrin (30 mg/mL stock; Roche 10652202001)	250 μ L	15 μ g/mL
Chemically defined lipid concentrate (100 $\times$ stock; Invitrogen 11905-031)	5 mL	1 $\times$

Store the medium (final 500 mL) at 4°C and use within 1 mo.

^aBSA may need to be batch-tested.

Collagenase Type IV (1 mg/mL)

Dissolve 0.5 g of collagenase type IV (Invitrogen 17104-019) in 500 mL of EpiSC medium <R>. Filter, aliquot in 10 mL, and freeze at -80°C. Thaw each aliquot as needed and store it at 4°C (do not refreeze).

EpiSC + FGF2 Medium

Reagent	Stock concentration	Final concentration	Amount
FGF2 stock solution <R>	10 μ g/mL	5 ng/mL	5 μ L
EpiSC medium <R>			10 mL

Store at 4°C and use within 1 wk.

EpiSC Medium

Reagent	Amount	Final concentration
KnockOut D-MEM (Invitrogen 10829-018)	385 mL	
KnockOut Serum Replacement (Invitrogen 10828-028)	100 mL	20%
Nonessential amino acids (10 mM stock; Invitrogen 11140-050)	5 mL	0.1 mM
β -Mercaptoethanol (10 mM stock) ^a	5 mL	0.1 mM
GlutaMAX (200 mM stock; Invitrogen 35050-061)	5 mL	2 mM

Store the medium (final volume 500 mL) at 4°C and use within 1 mo.

^a β -Mercaptoethanol 10 mM stock is prepared from 14.3 M stock (Sigma-Aldrich M7522) by adding 7 μ L to 10 mL of PBS.

FGF2 Stock Solution (10 μ g/mL)

Reagent	Amount
Recombinant fibroblast growth factor-2 (FGF2; R&D Systems 233-FB-025)	25 μ g
BSA fraction V solution in PBS (0.1%, w/v) <R>	2.5 mL
Resuspend lyophilized FGF2 in BSA/PBS, mix well, aliquot 50 μ L, and freeze at -80°C. Thaw each aliquot as needed and store it at 4°C. Do not refreeze the thawed aliquot.	

Freezing Medium for Feeder Cell Culture (2×)

Reagent	Final concentration
Fetal bovine serum (FBS)	20%
DMSO	20%
KnockOut DMEM	60%
Prepare fresh for each use and keep on ice.	

Freezing Medium for Feeder-Free Culture (1×)

Reagent	Final concentration
DMSO	10%
KnockOut Serum Replacement (KSR) (Invitrogen)	90%
Prepare fresh for each use and keep on ice.	

Pancreatin/Trypsin Solution

Reagent	g/20 mL	Final concentration
Pancreatin	0.50	2.5%
Trypsin	0.10	0.5%
Polyvinylpyrrolidone (optional)	0.10	0.5%

Make in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Tyrode Ringer's saline <R>.

The suspension will be difficult to filter-sterilize through a 0.45- μm Millipore filter without low-speed centrifugation or prefiltering through a Whatman No. 1 filter. Store sterile in small aliquots at -20°C .

Modified from Levak-Svajger et al. (1969).

Phosphate-Buffered Saline (PBS)

Reagent	Amount to add (for 1× solution)	Final concentration (1×)	Amount to add (for 10× stock)	Final concentration (10×)
NaCl	8 g	137 mM	80 g	1.37 M
KCl	0.2 g	2.7 mM	2 g	27 mM
Na_2HPO_4	1.44 g	10 mM	14.4 g	100 mM
KH_2PO_4	0.24 g	1.8 mM	2.4 g	18 mM

If necessary, PBS may be supplemented with the following:

$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.133 g	1 mM	1.33 g	10 mM
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	0.10 g	0.5 mM	1.0 g	5 mM

PBS can be made as a 1× solution or as a 10× stock. To prepare 1 L of either 1× or 10× PBS, dissolve the reagents listed above in 800 mL of H_2O . Adjust the pH to 7.4 (or 7.2, if required) with HCl, and then add H_2O to 1 L. Dispense the solution into aliquots and sterilize them by autoclaving for 20 min at 15 psi (1.05 kg/cm²) on liquid cycle or by filter sterilization. Store PBS at room temperature.

ACKNOWLEDGMENTS

I.G.M. Brons at the University of Cambridge and P.J. Tesar at Case Western Reserve University shared protocols, comments, and photographs.

P. Rugg-Gunn

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Cold Spring Harb Protoc; doi: 10.1101/pdb.prot093971

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