

Chapter 2

Reversion of Mouse Postimplantation Epiblast Stem Cells to a Naïve Pluripotent State by Modulation of Signalling Pathways

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Abstract

Mouse postimplantation epiblast cultured in activin and basic fibroblast growth factor gives rise to continuously growing epiblast stem cells (EpiSCs) that share key properties with postimplantation epiblast, such as DNA methylation and an inactive X-chromosome. EpiSCs also show a distinct gene expression profile compared to embryonic stem cells (ESCs) derived from preimplantation blastocysts, and do not contribute efficiently to chimeras. EpiSCs can, however, revert to pluripotent ESC-like cells upon exposure to leukemia inhibitory factor–Stat3 signalling on feeder cells. Here we describe a protocol for the establishment of EpiSCs and their reversion to ESCs.

Key words Epiblast stem cells, Embryonic stem cells, Postimplantation epiblast, Naïve pluripotency, Epigenetic reprogramming, LIF–Stat3 signalling

1 Introduction

The transition from preimplantation to postimplantation development is accompanied by extensive morphological and molecular changes of the rodent embryo [1]. All somatic cell lineages as well as the germ cells originate from the epiblast that transforms into a cup-shaped epithelium, acquires DNA methylation, and undergoes X-chromosome inactivation in female embryos [1–3]. Mouse postimplantation epiblast cultured in activin A and basic fibroblast growth factor (bFGF) gives rise to self-renewing cell lines termed epiblast stem cells (EpiSCs) [4, 5]. EpiSCs express the core pluripotency factors Oct4, Sox2, and Nanog and show multi-lineage differentiation potential in teratoma assays, but unlike embryonic stem cells (ESCs) derived from preimplantation blastocysts, they do not contribute efficiently, if at all, to chimeras [5, 6]. EpiSCs share key properties with postimplantation epiblast including the use of the proximal enhancer to drive *Oct4* expression instead of

the distal enhancer as in ESCs and primordial germ cells [6–8]. In addition, some pluripotency and germ cell-associated genes such as *Stella* and *Rex1* are methylated in EpiSCs [7].

We have previously shown that EpiSCs can revert to pluripotent ESC-like cells upon exposure to leukemia inhibitory factor (LIF)–Stat3 signalling on feeder cells [7]. Loss of DNA methylation, activation of the distal *Oct4* enhancer, X-chromosome reactivation, and re-expression of *Stella* and *Rex1* occur during the reversion process [7]. Here we describe a protocol for reversion of EpiSCs to ESC-like cells, termed reverted ESCs (rESCs). We first discuss the establishment of primary mouse embryonic fibroblasts (MEFs) for the preparation of feeder cells. We then explain how to derive EpiSCs from embryonic day (E) 5.5–6.5 postimplantation epiblast and we finally provide a detailed protocol for the reversion of EpiSCs to rESCs.

2 Materials

2.1 Cell

Culture Media

1. Sterile Dulbecco's modified Eagle medium/Ham's Nutrient Mixture F-12 (DMEM/F-12, 1:1, Gibco).
2. Sterile MEF medium: DMEM supplemented with 10 % (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. Mix 440 mL of DMEM with 50 mL of FCS, 5 mL of L-glutamine (200 mM stock solution), and 5 mL of penicillin/streptomycin solution (10,000 units/mL and 10,000 µg/mL stock solution). Store at 4 °C for up to 2 weeks.
3. Sterile EpiSC medium: Ham's Nutrient Mixture F-12 with GlutaMAX/Iscove's modified Dulbecco's medium (IMDM) (1:1) supplemented with 0.5 % (w/v) bovine serum albumin (BSA), 450 µM monothioglycerol, 7 µg/mL insulin, 15 µg/mL transferrin, 1× chemically defined lipid concentrate, 100 units/mL penicillin, 100 µg/mL streptomycin, 20 % (v/v) knockout serum replacement, 20 ng/mL activin A, and 12 ng/mL bFGF. Dissolve 2.5 g of BSA (Europa Bioproducts) in 250 mL of Ham's Nutrient Mixture F-12 with GlutaMAX, 250 mL of IMDM, 20 µL of monothioglycerol (≥97 %), 140 µL of human insulin (25 mg/mL stock solution), 75 µL of bovine apo-transferrin (100 mg/mL stock solution), 5 mL of chemically defined lipid concentrate (100× stock solution), and 5 mL of penicillin/streptomycin solution (10,000 units/mL and 10,000 µg/mL stock solution). Filter through a 0.45 µm membrane and store at 4 °C for up to 2 weeks. Before use, add 2 mL of knockout serum replacement, 4 µL of human activin A (50 µg/mL stock solution), and 2.4 µL of bFGF (50 µg/mL stock solution) to 8 mL of medium.

4. Sterile ESC medium: DMEM/F-12 (1:1) supplemented with 2 mM L-glutamine, nucleosides (30 μ M adenosine, 30 μ M guanosine, 30 μ M cytidine, 30 μ M uridine, 10 μ M thymidine), 0.1 mM MEM nonessential amino acids (NEAA), 1 mM sodium pyruvate, 1.2 mg/mL sodium bicarbonate, 0.05 mM 2-mercaptoethanol, 100 units/mL penicillin, 100 μ g/mL streptomycin, 20 % (v/v) FCS, and 1,000 units/mL LIF. Combine 367 mL of DMEM/F-12 (1:1) with 5 mL of L-GLUTAMINE (200 mM stock solution), 5 mL of nucleosides (100 $\times$ stock solution, *see Note 1*), 5 mL of MEM NEAA (10 mM stock solution), 5 mL of sodium pyruvate (100 mM stock solution), 8 mL of sodium bicarbonate (7.5 % stock solution), 500 μ L of 2-mercaptoethanol (50 mM stock solution), and 5 mL of penicillin/streptomycin solution (10,000 units/mL and 10,000 μ g/mL stock solution). Store at 4 $^{\circ}$ C for up to 4 weeks. Before use, add 2 mL of FCS (*see Note 2*) and 1 μ L of LIF (10,000,000 units/mL stock solution, ESGRO, Chemicon) to 8 mL of medium. Upon addition of FCS and LIF, store medium at 4 $^{\circ}$ C for up to 1 week.

2.2 Buffers and Solutions

1. Sterile phosphate-buffered saline (PBS) pH 7.4, calcium/magnesium-free.
2. Sterile distilled water (dH₂O).
3. Sterile 0.1 % (w/v) gelatin solution: Dissolve 0.1 g gelatin in 100 mL dH₂O in a 55 $^{\circ}$ C water bath. Autoclave and store at room temperature for up to 4 weeks.
4. Sterile collagenase solution: Knockout DMEM supplemented with 10 % (v/v) knockout serum replacement, 0.1 % (w/v) collagenase, 2 mM L-glutamine, 0.1 mM MEM NEAA, and 0.35 μ M 2-mercaptoethanol. Dissolve 500 mg of Collagenase Type IV (Invitrogen) in 400 mL of Knockout DMEM (Gibco) supplemented with 100 mL of knockout serum replacement (Gibco), 5 mL of L-glutamine (200 mM stock solution), 5 mL of MEM NEAA (10 mM stock solution), and 3.5 μ L of 2-mercaptoethanol (50 mM stock solution). Filter through a 0.45 μ M membrane and store at 4 $^{\circ}$ C for up to 1 week.
5. Sterile 0.05 % (w/v) trypsin/EDTA (1 $\times$) solution.
6. Sterile trypsin/pancreatin solution: 0.5 % (w/v) trypsin and 2.5 % (w/v) pancreatin in calcium/magnesium-free Tyrode Ringer's Saline pH 7.6–7.7. Dissolve 0.1 g trypsin and 0.5 g pancreatin in 20 mL of Tyrode Ringer's Saline (*see Note 3*). Filter through a 0.45 μ M membrane and store 1 mL aliquots at –20 $^{\circ}$ C.
7. Sterile mitomycin C solution (1 mg/mL in PBS): Store at 4 $^{\circ}$ C protected from light for up to 1 week or at –80 $^{\circ}$ C for up to 1 year.
8. Sterile dimethylsulfoxide (DMSO).

2.3 Equipment

1. Two pairs of forceps (Dumont #5).
2. Fine scissors.
3. Surgical blade.
4. Borosilicate glass capillaries (1.0 mm outside diameter, 0.58 mm inside diameter, Sutter Instruments).
5. Micropipette puller (Sutter Instruments).
6. Mouth pipette (*see* **Note 4**).
7. Stereomicroscope.
8. Sterile 90 mm bacteriological dishes.
9. Sterile 19 G needles.
10. Sterile 5 mL syringes.
11. Sterile 0.45 μ M filters.
12. Sterile 90 mm tissue culture dishes.
13. Sterile 24-well (1.9 cm²), 12-well (3.5 cm²), 6-well (9.6 cm²), and 4-well (1.9 cm²) multidishes.
14. Sterile 30 mL polypropylene tubes.
15. Sterile 25, 10, and 5 mL pipettes.
16. Sterile cryovials.
17. Cryo container.
18. Benchtop centrifuge.
19. Cell culture incubator: Sterile, humidified, 37 °C, 5 % CO₂, 95 % air.
20. Tissue culture hood.
21. Liquid nitrogen tank and –80 °C freezer.
22. Water bath.
23. Hot plate.
24. Vacuum pump.
25. Fluorescence microscope.

2.4 Animals

1. Pregnant female mice sacrificed humanely at 13.5 days of gestation for MEF derivation.
2. Pregnant female mice sacrificed humanely at 5.5–6.5 days of gestation for EpiSC derivation (preferably with *Oct4*- Δ PE-GFP reporter, *see* **Note 5**) [7, 8].

3 Methods

3.1 Establishment of Primary MEFs for Preparation of Feeder Cells

1. Prepare sterile MEF medium.
2. Open the abdominal cavity and remove the uterus from one to two pregnant female mice at 13.5 days of gestation.

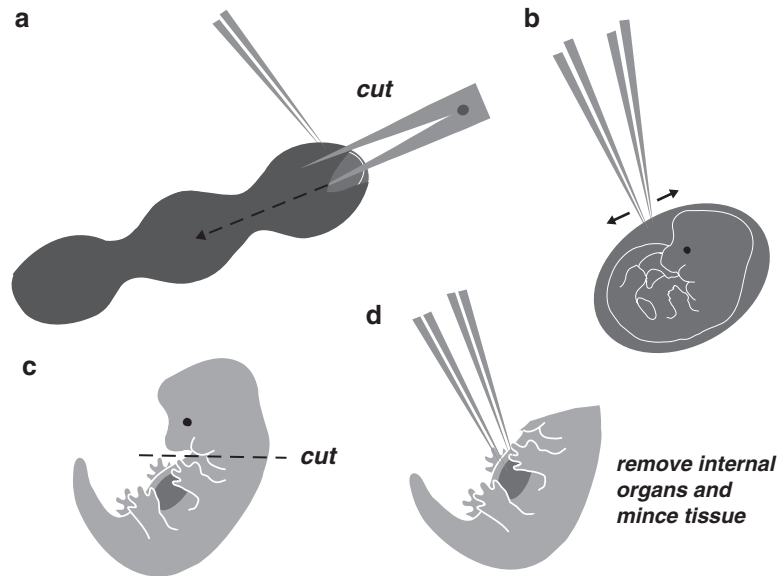


Fig. 1 Derivation of primary MEFs from E13.5 embryos. The uterine wall is cut (a) and the extraembryonic membranes are removed (b). Following removal of the head and the internal organs (c, d), the tissue is minced, transferred to a dish containing sterile MEF medium, and incubated in a humidified incubator at 37 °C and 5 % CO₂

3. Place the uterus into a 90 mm bacteriological dish containing 10 mL of sterile PBS and cut the uterine wall to peel out the deciduae (Fig. 1a).
4. Use two pairs of forceps to cut the Reichert's membrane and the placenta (Fig. 1b). Remove the extraembryonic membranes and transfer the embryos to a new 90 mm bacteriological dish containing 10 mL of sterile PBS.
5. Cut off the head (Fig. 1c) and remove the internal organs using a pair of forceps (Fig. 1d). Discard both head and internal organs and transfer the embryos to a new 90 mm bacteriological dish containing 10 mL of sterile PBS.
6. Mince the embryos into small pieces by passing them through a sterile 19 G needle three times in sterile PBS (*see Note 6*). Perform this step and all subsequent steps in an aseptic environment in a tissue culture hood to prevent contamination.
7. Transfer the minced embryo/PBS solution from a pool of three embryos (around 2 mL, *see Note 6*) to a 90 mm tissue culture dish containing 8 mL of sterile MEF medium.
8. Incubate the dish in a humidified incubator at 37 °C and 5 % CO₂ overnight.
9. Carefully replace the medium with 10 mL of fresh sterile MEF medium on the next day (*see Note 7*).

10. Incubate the dish in a humidified incubator at 37 °C and 5 % CO₂ overnight.
11. Passage the primary MEFs at a split ratio of 1:3 (*see Note 8*) on the next day: Wash the cells twice in PBS and add 3 mL of pre-warmed 0.05 % trypsin/EDTA solution (*see Note 9*) to a 90 mm tissue culture dish. Incubate the dish in a humidified incubator at 37 °C for 5 min. Tap the dish to confirm cell detachment and add 6 mL of sterile MEF medium. Transfer the cell suspension to a sterile 30 mL polypropylene tube and spin the cells down at 140×*g* for 3 min at room temperature. Aspirate the supernatant, resuspend the cell pellet in sterile MEF medium (10 mL for a 90 mm tissue culture dish), and seed MEFs onto a gelatin-coated 90 mm tissue culture dish (*see Note 10*). Incubate the dish in a humidified incubator at 37 °C and 5 % CO₂ overnight.
12. Expand MEFs to around passage 5 (*see Note 11*): When the culture reaches around 90 % confluence (every 3–4 days), passage the cells at a split ratio of 1:3 as in **step 11** above.
13. Treat a 90 mm tissue culture dish of MEFs (around 90 % confluence) at around passage 5 with 10 µg/mL mitomycin C: Combine 50 µL of sterile mitomycin C solution (1 mg/mL in PBS) with 5 mL of sterile MEF medium. Add this solution to a 90 mm dish of MEFs following aspiration of the culture medium. Incubate the dish in a humidified incubator at 37 °C and 5 % CO₂ for 2 h (*see Note 12*).
14. Freeze mitomycin C-treated MEFs (“feeder cells”): After 2 h of mitomycin C treatment, detach the cells as in **step 11** above. Gently resuspend the cell pellet in sterile freezing medium consisting of 10 % (v/v) DMSO, 10 % (v/v) FCS, and 80 % (v/v) MEF medium and quickly transfer the cell suspension into a labelled sterile cryovial. Freeze a 90 mm tissue culture dish of MEFs in 1 mL of freezing medium in one cryovial. Quickly place the cryovials into a cryo container and transfer to a –80 °C freezer overnight. Transfer the cryovials to a liquid nitrogen tank on the next day.

3.2 Derivation of EpiSCs from E5.5– E6.5 Postimplantation Epiblast

1. Thaw a vial of feeder cells into a 24-well dish 2 days prior to epiblast dissection: Place the cryovial into a 37 °C water bath until the ice dissolves. Quickly transfer the cell suspension into a 30 mL polypropylene tube containing 8 mL of sterile MEF medium and centrifuge at 140×*g* for 3 min at room temperature. Resuspend the cell pellet in 24 mL of sterile MEF medium and add 1 mL of cell suspension to each well of a 24-well dish (final feeder cell density: 3.5–4×10⁴ cells per cm²). Incubate the dish in a humidified incubator at 37 °C and 5 % CO₂ overnight. Replace the medium in each well with 1 mL of fresh sterile MEF medium on the next day. Incubate the dish in a humidified incubator at 37 °C and 5 % CO₂ until use.

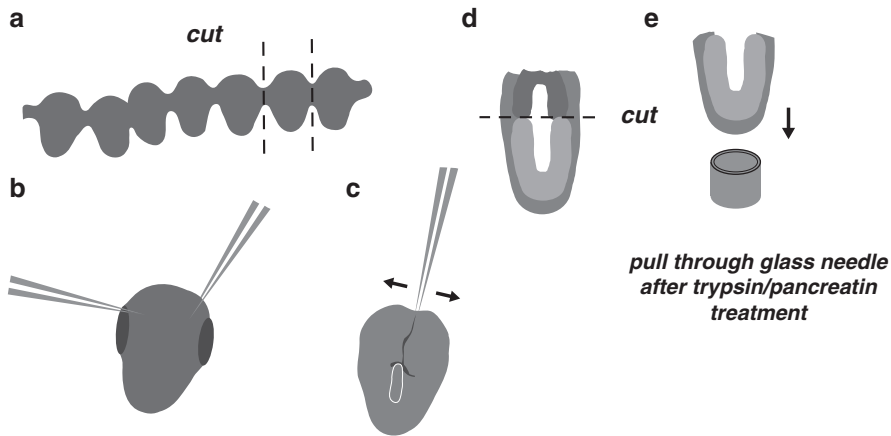


Fig. 2 Derivation of EpiSCs from E5.5–E6.5 postimplantation epiblast. The uterine wall between individual deciduae is cut (**a**), the embryo is dissected out of the decidua (**b**, **c**), and the trophoctoderm is separated from the epiblast/visceral endoderm tissue (**d**). Next, the epiblast/visceral endoderm tissue is pulled through a fine glass needle following trypsin/pancreatin treatment to remove the visceral endoderm from the epiblast (**e**). Finally, the dissected epiblast is transferred to a dish containing sterile EpiSC medium and feeder cells and incubated in a humidified incubator at 37 °C and 5 % CO₂

2. Prepare fine glass needles of various diameters (90–250 µm) by pulling borosilicate glass capillaries and cutting the end (*see Note 13*).
3. Prepare sterile EpiSC medium on the day of epiblast dissection. Wash the wells of a 24-well dish containing feeder cells (*see step 1* above) once with PBS and add 500 µL of sterile EpiSC medium to each well.
4. Open the abdominal cavity and remove the uterus from two to three pregnant female mice at 5.5–6.5 days of gestation (preferably with *Oct4* ΔPE-GFP reporter; *see Note 5*).
5. Place the uterus into a 90 mm bacteriological dish containing 10 mL of sterile MEF medium and cut the uterine wall between individual deciduae (Fig. 2a).
6. Open up the deciduae using a pair of forceps and carefully dissect out the embryos (Fig. 2b, c).
7. Transfer the embryos to a new 90 mm bacteriological dish containing 5 mL of sterile MEF medium.
8. Separate the trophoctoderm from the epiblast/visceral endoderm tissue using a surgical blade (Figs. 2d and 3a, b).
9. Discard the trophoctoderm and wash the epiblast/visceral endoderm tissue once in sterile PBS (*see Note 14*).
10. Place the epiblast/visceral endoderm tissue in sterile trypsin/pancreatin solution for 8 min at room temperature (*see Note 14*).
11. Transfer the epiblast/visceral endoderm tissue to a well of a 4-well dish containing 1 mL of EpiSC medium (*see Note 14*).

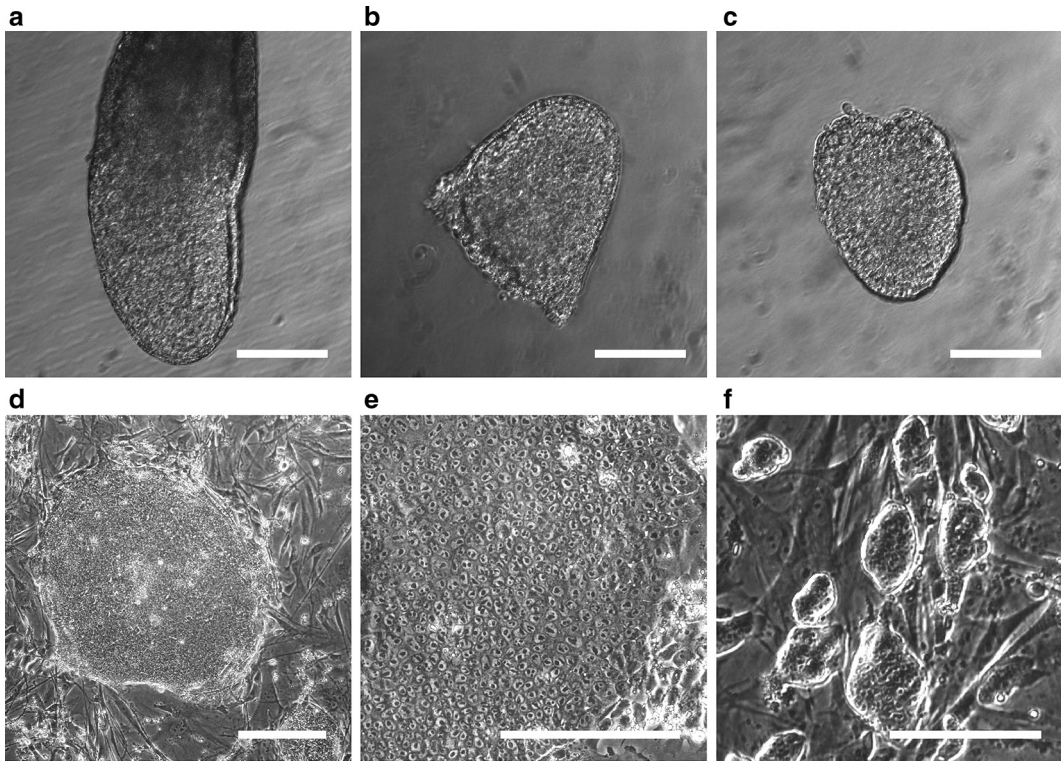


Fig. 3 Morphology of postimplantation epiblast, EpiSCs, and rESCs. Phase contrast images show an embryo at E6.5 (a), an embryo at E6.5 with the trophectoderm removed (b), and an embryo at E6.5 with both trophectoderm and visceral endoderm removed (c). Additional images show EpiSCs derived from E6.5 epiblast and maintained in EpiSC medium (activin A, bFGF, knockout serum replacement) on feeder cells (d, e), and rESCs grown in MEF medium (FCS, LIF) on feeder cells (f). Scale bar: 100 μ m

12. Pull the epiblast/visceral endoderm tissue through a fine glass needle to remove the visceral endoderm from the epiblast (*see Note 15*, Fig. 2c, e).
13. Discard the visceral endoderm and transfer the dissected epiblast into a well of a 24-well dish containing 500 μ L of sterile EpiSC medium and feeder cells (*see steps 1 and 3* above).
14. Incubate the dish in a humidified incubator at 37 $^{\circ}$ C and 5 % CO₂ overnight.
15. Replace the medium with 500 μ L of fresh sterile EpiSC medium every day. In addition, monitor the colony size and morphology every day (*see Note 16*). Perform this step and all subsequent steps in an aseptic environment in a tissue culture hood to prevent contamination.
16. Passage the primary colony after 5–10 days (*see Note 17*) at a split ratio of 1:1. Gently aspirate the culture medium and add 500 μ L of collagenase solution (pre-warmed to room temperature) to a well of a 24-well dish. Incubate the colony in collagenase for 6–8 min at room temperature. In the meantime

wash a well of a 24-well dish containing feeder cells (*see step 1* above) once with PBS and change the medium to 500 μ L of fresh sterile EpiSC medium. After 6–8 min of incubation in collagenase, carefully wash the colony twice in sterile DMEM/F-12 basal medium and add 500 μ L of sterile EpiSC medium to the well. Pipet up and down using a 200 μ L pipette under a stereomicroscope to break the colony into small clumps (*see Note 18*). Transfer the clumps into a well of a 24-well dish containing 500 μ L of EpiSC medium and feeder cells. Incubate the dish in a humidified incubator at 37 °C and 5 % CO₂ overnight.

17. Replace the medium with 500 μ L of fresh sterile EpiSC medium every day (*see Note 19*). In addition, monitor the colony size and morphology every day (*see Note 20*, Fig. 3d, e).
18. Passage the cells after 3–6 days at a split ratio of 1:2–1:4 as in **step 16** above.
19. Expand the cells to a 6-well dish (*see Note 21*) and freeze several vials of EpiSCs in sterile freezing medium consisting of 10 % (v/v) DMSO and 90 % (v/v) knockout serum replacement: Detach the cells as in **step 16** above (use 1.5 mL of collagenase per well of a 6-well dish), transfer the dissociated EpiSC colonies into a 30 mL polypropylene tube containing 8 mL of sterile DMEM/F-12 basal medium, and spin the cells down at 140 $\times g$ for 3 min at room temperature. Gently resuspend the cell pellet in freezing medium and transfer the cell suspension into a labelled sterile cryovial. Freeze one well of EpiSCs in a 6-well dish in 0.5 mL of freezing medium in one cryovial. Place the cryovials into a cryo container and transfer to a –80 °C freezer overnight. Transfer the cryovials to a liquid nitrogen tank on the next day. The content of one cryovial should be thawed into one well of a 6-well dish (*see Note 22*).

3.3 Reversion of EpiSCs to rESCs Upon Exposure to LIF/ Stat3 Signalling on Feeder Cells

1. Thaw a vial of feeder cells into several wells of a 12-well dish 5–7 days prior to the reversion experiment (*see Note 23*): Place the vial into a 37 °C water bath until the ice dissolves. Quickly transfer the cell suspension into a 30 mL polypropylene tube containing 8 mL of sterile MEF medium and centrifuge at 140 $\times g$ for 3 min at room temperature. Resuspend the cell pellet in sterile MEF medium and add 2 mL of the cell suspension per well of a 12-well dish. Incubate the dish in a humidified incubator at 37 °C and 5 % CO₂ overnight. Replace the medium in each well with 2 mL of fresh sterile MEF medium on the next day. Incubate the dish in a humidified incubator at 37 °C and 5 % CO₂ until use.
2. Prepare sterile ESC medium on the day of the reversion experiment. Wash a well of a 12-well dish containing feeder

cells (*see* **step 1** above) once with PBS and change the medium to 2 mL of sterile ESC medium.

3. Detach a sub-confluent well of EpiSCs (preferably with *Oct4-ΔPE-GFP* reporter, *see* **Note 5**) in a 12-well dish using 1 mL of collagenase as in **step 16** in Subheading 3.2.
4. Transfer the dissociated EpiSC colonies into a 30 mL polypropylene tube containing 8 mL of sterile DMEM/F-12 basal medium and spin the cells down at $140\times g$ for 3 min at room temperature. Aspirate the supernatant and resuspend the colonies in 800–1,000 μL of sterile ESC medium. Transfer 100 μL of cell suspension into a well of a 12-well dish (*see* **Note 24**) containing 2 mL of sterile ESC medium and 5–7-day-old feeder cells (*see* **steps 1** and **2** above).
5. Incubate the dish in a humidified incubator at 37 °C and 5 % CO_2 overnight.
6. Replace the medium with 2 mL of fresh sterile ESC medium every day for about 15–25 days.
7. After 5 days of reversion, check colonies for GFP expression in a fluorescent microscope every day. GFP-positive colony patches typically appear on days 10–20 (*see* **Note 25**). Depending on the growth rate, it may be necessary to passage the cells once prior to appearance of GFP-positive cells (on days 7–9), using collagenase as in **step 16** in Subheading 3.2 and at a split ratio of around 1:2.
8. Pick colony patches with more than 200 GFP-positive cells after 15–25 days of reversion using a fine glass needle (*see* **Note 26**) under the fluorescent microscope.
9. Transfer the GFP-positive colony patches to a new well of a 12-well dish containing 2 mL of sterile ESC medium and 5–7-day-old feeder cells (*see* **steps 1** and **2** above).
10. Incubate the dish in a humidified incubator at 37 °C and 5 % CO_2 overnight.
11. Replace the medium with 2 mL of fresh sterile ESC medium every day.
12. Thaw a vial of feeder cells into several wells of a 6-well dish (3 mL of MEF medium per well) as in **step 1** above.
13. Dissociate GFP-positive colonies (100–200 μm diameter) to a single-cell suspension using 0.05 % trypsin/EDTA 3–5 days after colony picking (*see* **steps 8** and **9** above): Wash the cells twice in PBS and add 500 μL of pre-warmed trypsin/EDTA solution (*see* **Note 9**) to a well of a 12-well dish. Incubate the dish in a humidified incubator at 37 °C for 8–10 min. Tap the dish to confirm cell detachment and add 1 mL of sterile MEF medium to the dish to inactivate the trypsin. Transfer the cell suspension into a sterile 30 mL polypropylene tube containing

5 mL of sterile MEF medium and spin the cells down at $140 \times g$ for 3 min at room temperature. Aspirate the supernatant and resuspend the cell pellet in 1 mL of sterile ESC medium. Transfer the cell suspension to a well of a 6-well dish containing feeder cells (*see* **step 12** above) and 3 mL of sterile ESC medium. Incubate the dish in a humidified incubator at 37 °C and 5 % CO₂ overnight. These cells can now be expanded as rESCs (*see* **Note 27**, Fig. 3f).

14. Freeze rESCs in sterile freezing medium containing 10 % (v/v) DMSO, 10 % (v/v) FCS, and 80 % (v/v) ESC medium: Detach the cells as in **step 13** above, gently resuspend the cell pellet in freezing medium, and transfer the cell suspension into a labelled cryovial. Freeze one well of rESCs in a 6-well dish in 1 mL of freezing medium in one cryovial. Place the cryovials into a cryo container and transfer to a -80 °C freezer overnight. Transfer the cryovials to a liquid nitrogen tank on the next day.

4 Notes

1. Dissolve 80 mg of adenosine, 85 mg of guanosine, 73 mg of cytidine, 73 mg of uridine, and 24 mg of thymidine (all from Sigma) in 100 mL of dH₂O by heating on a hot plate to about 40 °C. Filter through a 0.45 µm membrane, aliquot, and store at -20 °C for up to 1 year.
2. Test several batches of FCS for ESC derivation and culture, since batches vary in quality. Select the batch with the highest ESC derivation efficiency for use in EpiSC reversion experiments.
3. Calcium/magnesium-free Tyrode Ringer's Saline pH 7.6–7.7 is composed of 136.9 mM NaCl, 4 mM KCl, 0.15 mM NaH₂PO₄·5H₂O, 0.2 mM KH₂PO₄, 11.9 mM NaHCO₃, and 11 mM glucose. Dissolve 8 g NaCl, 0.3 g KCl, 0.093 g NaH₂PO₄·5H₂O, 0.025 g KH₂PO₄, 1 g NaHCO₃, and 2 g glucose in 1,000 mL of dH₂O. Adjust pH to 7.6–7.7.
4. A mouth pipette consists of an aspirator mouthpiece, tubing, and a Pasteur pipette [9]: Pull a Pasteur pipette on a flame to create a narrow opening. Attach the Pasteur pipette to a 1,000 µL tip with an inserted cotton plug and connect it to a mouthpiece through tubing. Insert a sterile 0.45 µm filter in the middle part of the tubing. As an alternative to a Pasteur pipette, a fine glass needle (*see* **Note 13**) can be mounted onto the mouth pipette.
5. The *Oct4*-ΔPE-GFP reporter enables rapid identification of rESCs, since GFP expression reflects activation of the distal *Oct4* enhancer that is inactive in EpiSCs and gets activated upon reversion to rESCs [7, 8]. Note that the genetic background of the mice used for EpiSC derivation has an impact on

derivation efficiency, quality of EpiSC lines, and their efficiency of reversion to rESCs. The use of a permissive background such as 129 enables robust reversion of EpiSCs to rESCs [10].

6. Attach a sterile 19 G needle to a sterile 5 mL syringe. Remove the plunger and place three embryos in the syringe. Add 2 mL of sterile PBS and insert the plunger. Pass the embryo/PBS solution through the needle three times.
7. Carefully remove the supernatant and add MEF medium as gently as possible to avoid detachment of tissue pieces and thus low yield of primary MEFs.
8. Transfer the cells of one confluent 90 mm tissue culture dish to three new 90 mm dishes.
9. Pre-warm the trypsin/EDTA solution to 37 °C in a water bath, since incubation in cold solution results in slower cell detachment, longer incubation times, and potentially more cell death.
10. Coat a 90 mm tissue culture dish with 5 mL of sterile 0.1 % gelatin solution for 30–60 min at room temperature. Aspirate the gelatin solution and dry the dish before use.
11. Expand primary MEFs to a maximum of five passages for their use as feeder cells, because their quality decreases with increasing passage number. Old cells may produce different cytokines and display altered adhesive properties compared to young cells. Alternatively, primary MEFs can be frozen at an early passage (*see step 14* in Subheading 3.1) and stored in liquid nitrogen. The cells can be thawed anytime (*see step 1* in Subheading 3.2), expanded, and treated with mitomycin C (*see step 13* in Subheading 3.1). The mitomycin C-treated MEFs (“feeder cells”) can be used immediately or frozen in liquid nitrogen for later use.
12. Do not incubate MEFs in mitomycin C solution for more or less than 2 h. Longer and shorter incubation times result in high toxicity and incomplete inhibition of cell proliferation, respectively.
13. Pull borosilicate glass capillaries using a micropipette puller (settings for P-80 Brown-Flaming Micropipette Puller/Sutter Instruments: HEAT=550, PULL=50, VEL=50). Prepare glass needles of various diameters (90–250 µm) by cutting the end of the needles.
14. Use a mouth pipette (*see Note 4*) with a mounted glass needle (*see Note 13*; 250 µm diameter) to transfer the epiblast/visceral endoderm tissue to a well of a 4-well dish containing 1 mL of sterile PBS, trypsin/pancreatin solution, or EpiSC medium. Epiblasts tend to be sticky following removal of the extraembryonic tissues and they occasionally attach to the surface of the glass needle. To avoid loss of epiblasts during

the transfer, suck in enough liquid and transfer the epiblasts as quickly as possible.

15. Use a mouth pipette (*see Note 4*) with a mounted glass needle (*see Note 13*) to suck the epiblast/visceral endoderm tissue from the distal end into the narrow opening of the glass needle (Fig. 2e). Depending on the size of the epiblast/visceral endoderm tissue, use glass needles with a diameter of 90–250 μm . Start with a wide glass needle, e.g., 250 μm . If the visceral endoderm does not separate from the epiblast, repeat this step using a narrow glass needle, e.g., 90 μm . The use of a glass needle with a diameter that is less than that of the epiblast (Fig. 2e) facilitates removal of the visceral endoderm.
16. The dissected epiblast attaches to the feeder-coated surface of the dish overnight and spreads out quickly to form a primary colony. The primary colony is compact and flat, and the cells show a high nuclear-to-cytoplasmic ratio (Fig. 3e). Over the next few days the colony grows and cells at the edge and the center start to differentiate. Cell death is common at this stage.
17. Passage the primary colony when the cells in the center of the colony die or differentiate. Do not wait until the entire colony differentiates (i.e., colony gets less compact with individual cells flattening out).
18. An optimal clump size (200–400 cells per clump) is crucial for the success of the cultures. Unlike ESCs, EpiSCs are sensitive to dissociation into single cells. If the clumps are too small or too big, the cells will die or differentiate, respectively. As an alternative to pipetting, the primary colony can be cut into small pieces using a fine glass needle (*see Note 26*).
19. The high proliferation rate of EpiSCs necessitates a frequent change of culture medium. As an alternative to a daily medium change, the pH value of the medium can be used as an indicator: a yellow color of EpiSC medium (i.e., DMEM/F-12 with phenol red) indicates a drop in pH value, requiring a medium change. In addition, EpiSCs must be incubated in a saturated humidified atmosphere to minimize media evaporation.
20. EpiSCs form flattened, yet compact, colonies (Fig. 3d) and show a high nuclear-to-cytoplasmic ratio and prominent nucleoli (Fig. 3e) [4, 5]. Differentiated cells are usually present at the edge of the colony (Fig. 3d, e), especially at early passage numbers. The colony compactness and morphology of EpiSC lines vary and may be influenced by genetic background. The derivation efficiency of EpiSCs from E6.5 epiblast is generally around 70 % (i.e., 7 out of 10 explanted epiblasts give rise to a continuously growing EpiSC line).
21. Passage EpiSCs every 1–2 days at a split ratio of 1:2–1:4, since frequent passage and high plating densities minimize cell death

and differentiation. The growth rate of different EpiSC lines varies slightly and may depend on genetic background. EpiSCs can be maintained on feeder cells or moved to feeder-free conditions on fibronectin. As an alternative to dissociation of EpiSC colonies with collagenase and pipetting, EpiSCs can be detached using accutase (incubation for 1–2 min at 37 °C). Since accutase treatment generates a near-single-cell suspension, but is less toxic than trypsin/EDTA, this method is particularly suitable for flow cytometric sorting and for feeder-free maintenance of EpiSCs. Once established, verify pluripotency gene expression of EpiSC lines by immunostaining with Oct4, Sox2, and Nanog antibodies [4]. Confirm the presence of nuclear foci of histone 3 lysine 27 trimethylation (H3K27me3), indicating X-chromosome inactivation, in female EpiSC lines by immunostaining with H3K27me3 antibodies [6]. Determine the karyotype of EpiSCs, e.g., by metaphase spreads, to detect chromosomal abnormalities [4]. Test the differentiation potential of EpiSCs by teratoma formation or directed in vitro differentiation assays [4, 5].

22. Place a cryovial of EpiSCs into a 37 °C water bath until the ice dissolves. Quickly transfer the cell suspension into a 30 mL polypropylene tube containing 8 mL of sterile DMEM:F-12 basal medium and centrifuge at $140\times g$ for 3 min at room temperature. Gently resuspend the cell pellet in 3 mL of sterile EpiSC medium and transfer the cell suspension to a well of a 6-well dish containing feeder cells after one wash in sterile PBS. Incubate the dish in a humidified incubator at 37 °C and 5 % CO₂ overnight. To maximize plating efficiency, EpiSCs should be plated at high density after thawing and passaged on the day after thawing.
23. An optimal feeder cell density ($7\text{--}7.5\times 10^4$ cells per cm²) is crucial for the success of the reversion experiment. The feeder cell density should be higher than the density used for EpiSC derivation.
24. Plate EpiSC colonies at low density, since the reversion process is slow (15–25 days). A subconfluent well of EpiSCs in a 12-well dish should be seeded in 8–10 wells of a 12-well dish (i.e., split ratio of 1:8–1:10).
25. The success, rate, and efficiency of reversion of EpiSCs to rESCs depend on several factors, such as the quality of the FCS batch (*see Note 2*), the quality of feeder cells (*see Note 11*), the density of feeder cells (*see Note 23*), the quality of EpiSCs (*see Notes 20 and 21*), the density of EpiSCs (*see Note 24*), and the genetic background (*see Note 5*). In general, reversion of EpiSCs to rESCs takes between 2 and 3 weeks and several GFP-positive colony patches appear per well of a 12-well dish.

26. Pull borosilicate glass capillaries using a micropipette puller (settings for P-80 Brown-Flaming Micropipette Puller/Sutter Instruments: HEAT = 550, PULL = 50, VEL = 50), but do not cut the end of the glass needles for this application.
27. The growth rate and morphology of rESCs are similar to mouse ESCs: Passage rESCs every 2–3 days at a split ratio of 1:10–1:20. The cells show a dome-shaped morphology (Fig. 2.3f) with homogeneous *Oct4*- Δ PE-GFP expression [7] when grown in MEF medium (FCS and LIF) on feeder cells. Verify pluripotency gene expression of rESCs by immunostaining with Oct4, Sox2, and Nanog antibodies and confirm the loss of nuclear foci of H3K27me3, that indicates X-chromosome reactivation, in female rESCs by immunostaining with H3K27me3 antibodies [7]. Inject 15–20 *Oct4*- Δ PE-GFP rESCs into E3.5 C57BL/6 blastocysts and assess chimerism by agouti coat color of pups [7]. Confirm germ line contribution by inspecting E13.5 C57BL/6 male and female genital ridges for GFP-positive cells and by assessing the coat color of progeny derived from chimeras [7].

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