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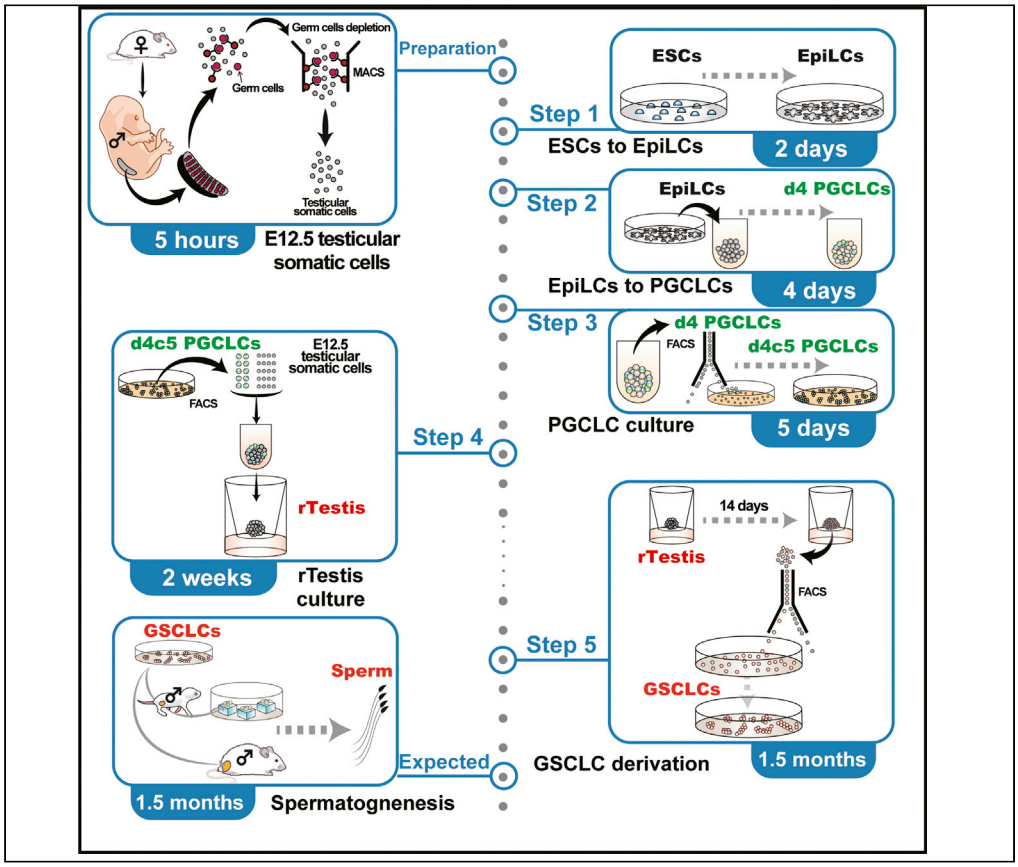
Optimized protocol to derive germline stem-cell-like cells from mouse pluripotent stem cells



Male germ-cell development comprises primordial germ-cell (PGC) development, spermatogonium differentiation, and ensuing spermatogenesis. We present a step-by-step protocol for differentiation of mouse pluripotent stem cells (PSCs) into germline stem-cell-like cells (GSCLCs) via PGC-like cell and spermatogonium-like cell intermediates. The differentiation protocol has higher fidelity than our previous protocol. Upon transplantation into testes *in vivo* or culture for testis transplants, GSCLCs robustly contribute to spermatogenesis, providing a paradigm for PSC-based reconstitution of mammalian male germ-cell development.

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

Yukiko Ishikura, Hiroshi Ohta, Masahiro Nagano, Mitinori Saitou

y.ishikura@anat2.med.kyoto-u.ac.jp (Y.I.)
saitou@anat2.med.kyoto-u.ac.jp (M.S.)

Highlights

Protocol for generating germline stem-cell-like cells (GSCLC) from mouse PSCs

GSCLCs bear robust spermatogenic potential

In vitro reconstitution of whole male germ-cell development in mammals

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Optimized protocol to derive germline stem-cell-like cells from mouse pluripotent stem cells

Yukiko Ishikura,[1,2,4,*] Hiroshi Ohta,[1,2] Masahiro Nagano,[1,2] and Mitinori Saitou[1,2,3,5,*]

[1]Institute for the Advanced Study of Human Biology (WPI-ASHBi), Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan

[2]Department of Anatomy and Cell Biology, Graduate School of Medicine, Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan

[3]Center for iPS Cell Research and Application (CiRA), Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

[4]Technical contact

[5]Lead contact

*Correspondence: y.ishikura@anat2.med.kyoto-u.ac.jp (Y.I.), saitou@anat2.med.kyoto-u.ac.jp (M.S.)
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SUMMARY

Male germ-cell development comprises primordial germ-cell (PGC) development, spermatogonium differentiation, and ensuing spermatogenesis. We present a step-by-step protocol for differentiation of mouse pluripotent stem cells (PSCs) into germline stem-cell-like cells (GSCLCs) via PGC-like cell and spermatogonium-like cell intermediates. The differentiation protocol has higher fidelity than our previous protocol. Upon transplantation into testes *in vivo* or culture for testis transplants, GSCLCs robustly contribute to spermatogenesis, providing a paradigm for PSC-based reconstitution of mammalian male germ-cell development.

For complete details on the use and execution of this protocol, please refer to Ishikura et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the steps (see Figure 1 for an overview) using a specific mouse embryonic stem cell (ESC) line bearing transgenes expressing membrane-targeted Venus (mVenus) under the control of *Blimp1* (also known as *Prdm1*), enhanced cyan fluorescent protein (ECFP) under the control of *Stella* (also known as *Dppa3*), and red fluorescent protein (RFP) under the control of *Ddx4* (also known as *Mvh*) (*Blimp1-mVenus*; *Stella-ECFP*; *Mvh-RFP* (BVSCVR)). *Blimp1-mVenus* (BV) and *Stella-ECFP* (SC) mark primordial germ cells (PGCs), while *Mvh-RFP* (VR) initiates expression after PGCs' colonization of embryonic gonads and male germ-cell specification. Other markers can be used to monitor the development of PGCs and male germ cells in culture. This protocol has higher fidelity than our previous protocol (Ishikura et al., 2016).

Institutional permissions

All the animal experiments must be performed in accordance with the ethical guidelines of the institution. All animal experiments in this protocol were performed under the ethical guidelines of Kyoto University (Approval no. MedKyo19001).

Preparation of stock solutions for cell culture

Timing: 2 days for steps 1–22

Timing: 3 h for steps 23–25

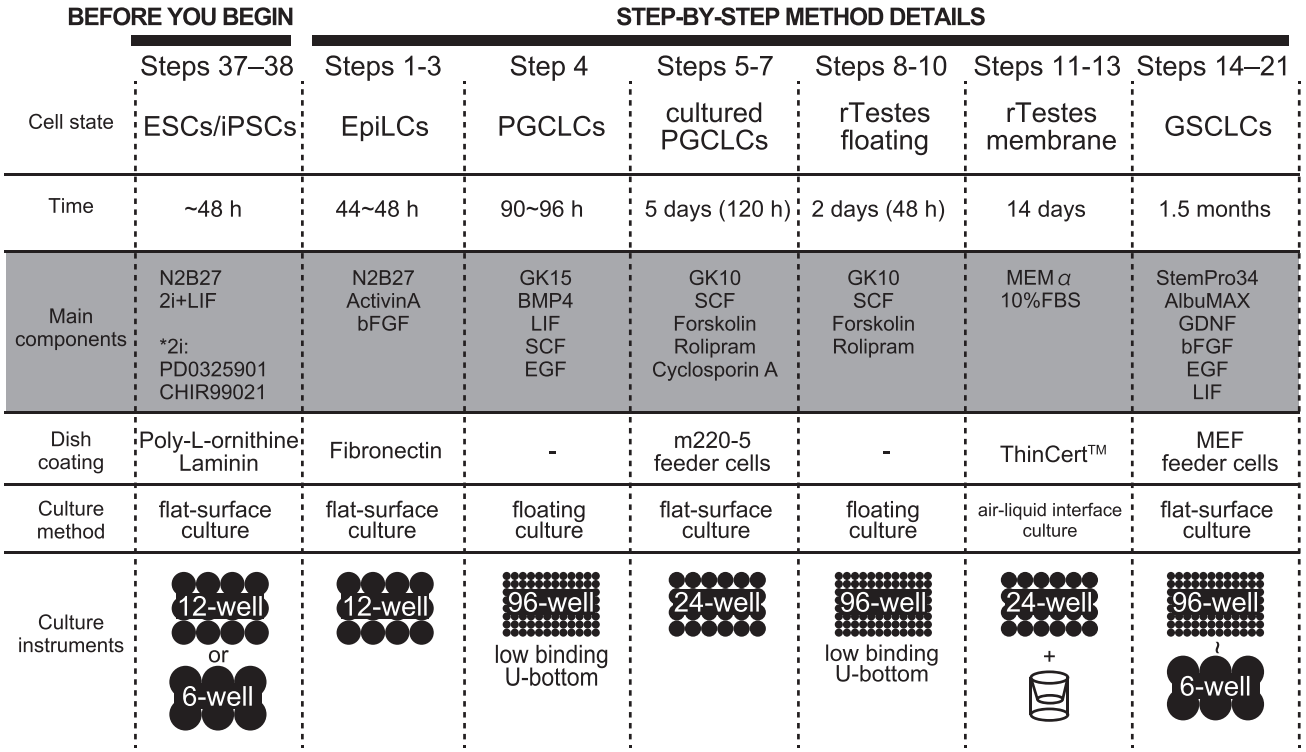

Figure 1. An overview of the protocol. An overview of the experiment for the derivation of GSCLCs

Prepare aliquots of stock solutions.

Unless otherwise stated, all medium preparations and culture operations are carried out on a clean bench. All centrifugation steps are carried out at 25°C. We do not pay a specific attention to the pH of the working solution/buffer, except the color of the phenol red included in the medium.

Storage and working concentration of reagents

Reagent	Storage concentration	Working concentration	Storage condition
FBS	Directly aliquoted	Depends on the culture medium	Store at −20°C
Insulin (powder)	25 mg/mL	2.5 mg/mL	Store at −80°C
Apo-Transferrin	100 mg/mL	10 mg/mL	Store at −80°C
Progesterone	0.6 mg/mL	2 μg/mL	Store at −20°C
Putrescine Dihydrochloride	160 mg/mL	1.6 mg/mL	Store at −20°C
Sodium Selenite	3 mM	3 μM	Store at −20°C
L-Glutamine	Directly aliquoted	1:100	Store at −20°C
GlutaMAX™-I	Directly aliquoted	1:100	Store at 4°C
Sodium Pyruvate	Directly aliquoted	1:100	Store at −20°C
Penicillin-Streptomycin Solution	Directly aliquoted	1:100	Store at −20°C
MEM Non-Essential Amino Acids Solution (NEAA)	Directly aliquoted	1:100	Store at −20°C
Knockout Serum Replacement (KSR)	Directly aliquoted	Depends on the culture medium	Store at −20°C
CHIR99021	30 mM	3 μM	Store at −80°C
PD0325901	10 mM	0.4 μM	Store at −80°C
FGF2	10 μg/mL	12 ng/mL	Store at −80°C
Activin A	50 μg/mL	20 ng/mL	Store at −80°C
BMP4	50 μg/mL	500 ng/mL	Store at −80°C

(*Continued on next page*)

Continued

Storage and working concentration of reagents

Reagent	Storage concentration	Working concentration	Storage condition
Stem Cell Factor (SCF)	50 μg/mL	100 ng/mL	Store at −80°C
EGF	500 μg/mL	50 ng/mL	Store at −80°C
LIF (ESGRO)	10^7 U/mL	10^3 U/mL	Store at 4°C
Forskolin	30 mM	10 μM	Store at −80°C
Rolipram	50 mM	10 μM	Store at −80°C
Cyclosporine A	50 mM	5 μM	Store at −20°C
GDNF	20 μg/mL	20 ng/mL	Store at −20°C
Poly-L-ornithine	0.01% (w/v)	0.01% (w/v)	Store at 4°C
Laminin	~2 mg/mL	10–300 ng/mL	Store at −80°C
Fibronectin	1 mg/mL	16.6 μg/mL	Store at 4°C
DNase I	20 mg/mL	0.1 mg/mL	Store at −20°C
0.1% Gelatin Solution	0.1% (v/w)	0.1% (v/w)	Store at 25°C

1. Prepare 2 mL aliquots of FBS, sodium pyruvate, penicillin-streptomycin solution, and MEM non-essential amino acids solution (NEAA), and store at −20°C.
2. Dissolve 100 mg of insulin (powder) in 4 mL of filter-sterilized 10 mM HCl at 4°C for at least 8 h to prepare a stock solution of 25 mg/mL insulin, aliquot, and store at −80°C.
3. Dissolve 100 mg of apo-transferrin in 1 mL of sterile distilled water at 4°C for at least 8 h to prepare a stock solution of 100 mg/mL apo-transferrin, aliquot, and store at −80°C.
4. Dissolve 6 mg of progesterone in 10 mL of ethanol to prepare a stock solution of 0.6 mg/mL progesterone, sterilize with a 0.22 μm filter, aliquot, and store at −20°C or below.
5. Dissolve 1.6 g of putrescine in 10 mL of sterile distilled water to prepare a stock solution of 160 mg/mL putrescine dihydrochloride, sterilize with a 0.22 μm filter, aliquot, and store at −20°C or below.
6. Dissolve 2.59 mg of sodium selenite in 5 mL of sterile distilled water to prepare a stock solution of 3 mM sodium selenite, sterilize with a 0.22 μm filter, aliquot, and store at −20°C or below.
7. Dissolve 5 mg of CHIR99021 in 358.2 μL of DMSO to prepare a stock solution of 30 mM CHIR99021, aliquot, and store at −80°C.
8. Dissolve 2 mg of PD0325901 in 414.7 μL of DMSO to prepare a stock solution of 10 mM PD0325901, aliquot, and store at −80°C.
9. Dissolve 10 μg of bFGF in 1 mL of filter-sterilized PBS (pH 7.2) containing 0.1% BSA to prepare a stock solution of 10 μg/mL bFGF, aliquot, and store at −20°C or below.
10. Dissolve 10 μg of activin A in 0.2 mL of filter-sterilized distilled water containing 0.1% BSA to prepare a stock solution of 50 μg/mL activin A, aliquot, and store at −80°C.
11. Dissolve 1 mg of BMP4 in 20 mL of filter-sterilized 4 mM HCl solution containing 0.1% BSA to prepare a stock solution of 50 μg/mL BMP4, aliquot, and store at −80°C.
12. Dissolve 500 μg of SCF in 10 mL of filter-sterilized PBS (pH 7.2) containing 0.1% BSA to prepare a stock solution of 50 μg/mL SCF, aliquot, and store at −80°C.
13. Dissolve 200 μg of EGF in 0.4 mL of filter-sterilized PBS (pH 7.2) containing 0.1% BSA to prepare a stock solution of 500 μg/mL EGF, aliquot, and store at −80°C.
14. Prepare 50 μL aliquots of LIF (ESGRO, 10^7 U/mL), and store at 4°C.
15. Dissolve 10 mg of forskolin in 811.7 μL of DMSO to prepare a stock solution of 30 mM forskolin, aliquot, and store at −80°C.
16. Dissolve 10 mg of rolipram in 726.4 μL of DMSO to prepare a stock solution of 50 mM rolipram, aliquot, and store at −80°C.
17. Dissolve 25 mg of cyclosporin A in 415.7 μL of DMSO to prepare a stock solution of 50 mM cyclosporin A, aliquot, and store at −20°C.
18. Dissolve 50 μg of GDNF in 2.5 mL of filter-sterilized PBS (pH 7.2) containing 0.1% BSA to prepare a stock solution of 20 μg/mL GDNF, aliquot, and store at −20°C or below.

19. Dissolve 50 mg of poly-L-ornithine in 500 mL of sterile distilled water to prepare a solution of 0.01% (w/v) poly-L-ornithine, and store at 4°C.
20. Dispense ~2 mg/mL laminin solution on ice and store at −80°C. After thawing, store at 4°C and handle on ice.
21. Dissolve 100 mg of DNase I in 5 mL of sterile distilled water to prepare a stock solution of 20 mg/mL DNase I, aliquot, and store at −20°C.
22. Dissolve 0.5 g of bovine gelatin in 500 mL of sterile distilled water to prepare 0.1% (w/v) gelatin solution, autoclave, and store at 25°C.

Preparation of N2B27 stock.

N2B27 is a serum-free medium for ESC culture. Two changes are made from the reported procedures (Nichols and Ying, 2006; Ying et al., 2008): we use DMEM/F12 without HEPES or phenol red, and we use B27 supplement without vitamin A.

23. Prepare N2 supplement 100× solution.

N2 supplement 100× solution		
Reagent	Final concentration	Amount
DMEM/F12 (HEPES and Phenol Red Free)	n/a	3,595 μL
Insulin	2.5 mg/mL	500 μL
Apo-Transferrin	10 mg/mL	500 μL
Progesterone	2 μg/mL	16.7 μL
Putrescine Dihydrochloride	1.6 mg/mL	50 μL
Sodium selenite	3 μM	5 μL
Bovine Albumin Fraction V	5 mg/mL	333.3 μL
Total	n/a	5 mL

24. Prepare Solution A and B for N2B27.
    a. Use N2 supplement 100 × solution immediately for the preparation of solution A. Also, prepare solution B.

Solution A for N2B27		
Reagent	Final concentration	Amount
DMEM/F12	n/a	495 mL
N2 Supplement 100× Solution	× 1	5 mL
2-Mercaptoethanol	0.1 mM	900 μL
Total	n/a	500.9 mL

Note: Prepare before use.

Solution B for N2B27		
Reagent	Final concentration	Amount
Neurobasal™ Medium minus Phenol Red	n/a	480 mL
B-27™ Supplement minus Vitamin A	n/a	10 mL
Penicillin-Streptomycin Solution	100 U/mL 100 g/mL	5 mL
L-Glutamine Solution	2 mM	5 mL
2-Mercaptoethanol	0.1 mM	900 μL
Total	n/a	500.9 mL

Note: Prepare before use.

25. Prepare N2B27.
    a. Mix 20 mL of solution A and B to make a total of 40 mL of N2B27 in a 50 mL tube.
    b. Store at −80°C. Once thawed, store at 4°C and use within 2 weeks.

N2B27		
Reagent	Final concentration	Amount
Solution A for N2B27	× 1	20 mL
Solution B for N2B27	× 1	20 mL
Total	n/a	40 mL

Note: Typically, we produce 4 liters of the medium, dispense it into 100 tubes (40 mL aliquots in 50 mL Falcon tubes), and store the tubes at −80°C until use.

Preparation of cell stocks

Timing: 4 weeks for steps 26–28

Timing: 2 weeks for steps 29–31

Timing: 5 h for steps 32–35

Timing: 1–2 weeks for steps 36–39

m220-5 feeders.

PGCLCs are expanded on m220-5 feeder cells, which are originally established as m220 cells from mutant mice lacking the steel factor gene and transfected with a gene expressing a membrane-bound form of the steel factor (Dolci et al., 1991; Majumdar et al., 1994; Matsui et al., 1991). The m220 cells are highly vulnerable to mitomycin C (MMC) treatment, and therefore, an MMC-resistant m220-5 subline was cloned, expanded, and stocked frozen. For a step-by-step protocol, see (Miyauchi et al., 2018; Ohta et al., 2017). Here, we describe a procedure for preparing MMC-treated stocks of m220-5 cells acclimated to forskolin and rolipram from the MMC-resistant m220-5 subline. We typically expand the MMC-resistant m220-5 cells to confluence in up to ~30–40 × 15 cm dishes to prepare 50 stock tubes.

26. Thaw the m220-5 cells.
    a. Add 3 mL of 0.1% gelatin solution to the 10 cm dish and incubate the dish for 30 min at 25°C.
    b. Thaw a frozen m220-5 stock tube (~6–8 × 10^5 cells/tube) quickly by incubating in a water bath at 37°C.
    c. Transfer the cells to a 15 mL tube containing more than 10 times the volume of the pre-warmed wash buffer.
    d. Centrifuge the tube at 220 × *g* (1,200 rpm with a typical bench-top centrifuge) for 3 min to pellet the cells.
    e. Remove the supernatant and resuspend the cells in 10 mL of serum medium.
    f. Aspirate the gelatin solution from the 10 cm dish.
    g. Transfer the m220-5 cell suspension to the 10 cm dish, and culture 1 or 2 days until the cells reach sub-confluence (~80% confluence).
    h. Replace the serum culture medium with forskolin/rolipram medium.
    i. Culture the cells at 37°C in 5% CO_2, 95% air until the cells reach sub-confluence (~90% confluence).
27. Passage the m220-5 cells in the forskolin/rolipram medium.

a. Aspirate the medium and wash the dishes containing sub-confluent m220-5 cells with PBS (5 mL/10 cm dish, 15 mL/15 cm dish).
b. Add 0.05% trypsin-EDTA/PBS (3 mL/10 cm dish, 5 mL/15 cm dish).
c. Incubate the cells at 37°C in 5% CO_2, 95% air for 5 min.
d. Tap the dish to detach the cells from the dish.
e. Add prewarmed serum medium (3 mL/10 cm dish, 5 mL/15 cm dish), dissociate the cells by gentle pipetting, and transfer the cells into a 50 mL tube.
f. Count the number of cells using a hemocytometer.
g. Centrifuge the cells at 220 × *g* for 3 min to pellet the cells.
h. Aspirate the supernatant, dislodge the cell pellet by gentle tapping, and resuspend the cells in the forskolin/rolipram medium (10 mL/10 cm dish, 20 mL/15 cm dish).
i. Transfer the cells to culture dishes (7×10^5 cells/10 mL/10 cm dish, 2×10^6 cells/20 mL/15 cm dish).
j. Incubate the cells at 37°C in 5% CO_2, 95% air.
k. Change half the volume of the forskolin/rolipram medium every 2 days (5 mL/10 cm dish, 10 mL/15 cm dish), and culture until the cells reach sub-confluence.
l. Passage the cells several times every ~4–7 days and scale up to sub-confluence in ~30–40 × 15 cm dishes.

Note: The m220-5 cells should be monitored carefully to avoid their overgrowth. The overgrown m220-5 cells show reduced capacity for PGCLC expansion.

28. Treat with MMC and prepare stocks of the forskolin/rolipram-resistant m220-5 cells.
    a. Dissolve 2 mg of MMC in 5 mL of serum medium and then add 45 mL of serum medium to prepare 40 μg/mL of the MMC solution.
    b. Quantify the medium volume in a dish by aspirating the medium with a 25 mL pipette and adjust the medium volume of the 15 cm dish with sub-confluent m220-5 cells to 15 mL.
    c. Add 375 μL of the 40 μg/mL MMC solution to the culture to make the final MMC concentration of the culture medium 1 μg/mL.
    d. Incubate the cells for 2 h at 37°C in 5% CO_2, 95% air.
    e. Aspirate the medium and wash the cells with 10 mL of PBS/15 cm dish three times.
    f. Add 20 mL of the serum medium/15 cm dish and culture the cells for ~5−6 h to recover their viability.
    g. Aspirate the medium and wash the cells with 10 mL of PBS/15 cm dish.
    h. Add 5 mL of 0.05% trypsin-EDTA/PBS/15 cm dish.
    i. Incubate the cells at 37°C in 5% CO_2, 95% air for 5 min.
    j. Tap the dish to detach the cells from the dish.
    k. Add 10 mL of the prewarmed serum medium/15 cm dish, dissociate the cells by gentle pipetting, and transfer the cells into 50 mL tubes.
    l. Add 200 μL DNase I in 40 mL of cell suspension and pipet well to mix.
    m. If cells are transferred into several 50 mL tubes, centrifuge the tubes at 220 × *g* for 5 min, aspirate the medium, dislodge the pellet by tapping, resuspend the cell pellet in ~0.5–1 mL of the serum medium, and combine the cells in one 50 mL tube.
    n. Count the number of cells using a hemocytometer.
    o. Centrifuge the cells at 220 × *g* for 5 min to pellet the cells.
    p. Aspirate the supernatant and dislodge the cell pellet by gentle tapping.
    q. Resuspend the cells in CELLBANKER I plus so that the cell concentration becomes ~$0.8–1.2 \times 10^7$ cells/mL and transfer 500 μL to each cryopreservation tube (~$4–6 \times 10^6$ cells/tube).
    r. Store the cells at −80°C (or lower temperature) and use within 6 months.

Note: In case the operation time is prolonged, the cell suspension should be kept on ice to enhance the cell viability.

0.05% trypsin-EDTA/PBS		
Reagent	Final concentration	Amount
0.5% Trypsin-EDTA	0.05%	1 mL
PBS	n/a	9 mL
Total	n/a	10 mL

Note: Prepare before use and prewarm.

Wash buffer		
Reagent	Final concentration	Amount
DMEM/F12	n/a	500 mL
7.5% BSA Fraction V	0.1%	6.7 mL
Total	n/a	506.7 mL

Note: Store up to 1 month at 4°C.

Serum medium		
Reagent	Final concentration	Amount
DMEM	n/a	500 mL
FBS [stock]	8.9%	50 mL
Penicillin-Streptomycin Solution [stock]	89 U/mL 89 g/mL	5 mL
GlutaMAX™-I	× 112 dilution	5 mL
Total	n/a	560 mL

Note: Store up to 1 month at 4°C.

Forskolin/Rolipram medium		
Reagent	Final concentration	Amount
DMEM	n/a	500 mL
FBS [stock]	8.9%	50 mL
Penicillin-Streptomycin Solution [stock]	89 U/mL 89 g/mL	5 mL
GlutaMAX™-I	× 112 dilution	5 mL
Forskolin [stock]	8.9 μM	165 μL
Rolipram [stock]	8.9 μM	100 μL
Total	n/a	560 mL

Note: Store up to 2 weeks at 4°C.

MMC solution		
Reagent	Temporary concentration	Amount
Mitomycin C	40 μg/mL	2 mg
Serum Medium	n/a	~50 mL
Total	n/a	50 mL

Note: Prepare before use.

Mouse embryonic fibroblasts (MEFs).

GSCLCs are derived and expanded on the MMC-treated MEFs. We usually use CD1 (ICR) MEFs from embryonic day (E)13.5 fetal trunks, passaged once, and frozen as a primary MEF frozen stock, according to the general step-by-step protocol (Behringer et al., 2014). We typically prepare the primary MEF frozen stocks from 10–12 fetuses at E13.5 up to ~50–70 tubes and prepare the MMC-MEF stocks from 1 primary MEF frozen stock up to ~60–80 plates of the 10 cm dishes to prepare ~150−200 tubes of frozen stock.

29. Thaw and expand MEFs.
    a. Thaw a primary MEF frozen stock tube (~4 × 10^6 cells/tube) quickly by incubating the tube in the 37°C water bath.
    b. Transfer the cells to a 15 mL tube containing more than 10 times the volume of wash buffer.
    c. Centrifuge the tube at 220 × *g* for 3 min to pellet the cells.
    d. Aspirate the supernatant and resuspend the cells in 4 mL of serum medium.
    e. Count the number of cells by a hemocytometer.
    f. Add 10 mL of the serum medium to 10 cm dishes.
    g. Transfer the MEF suspension to the dishes so that the cell concentration becomes ~1.0–1.2 × 10^6 cells/10 cm dish.
    h. Incubate the cells at 37°C in 5% CO_2, 95% air until the cells reach sub-confluence (~90% confluence: 2 or 3 days).
30. Passage MEFs.
    a. Aspirate the medium and wash the cells with PBS.
    b. Add 0.25% trypsin-EDTA (1 mL/10 cm dish).
    c. Incubate the cells at 37°C in 5% CO_2, 95% air for 3 min.
    d. Tap the dish to detach the cells from the dish.
    e. Add the prewarmed serum medium (4 mL/10 cm dish).
    f. Dissociate the cells by gentle pipetting and transfer the cells into a 50 mL tube.
    g. Count the number of cells by a hemocytometer.
    h. Centrifuge the cells at 220 × *g* for 3 min to pellet the cells.
    i. Aspirate the supernatant and dislodge the cell pellet by gentle tapping.
    j. Resuspend the cells in the serum medium and transfer the MEF suspension to the dishes so that the cell concentration becomes 0.7–1.0 × 10^6 cells/10 cm dish.

    ⚠ CRITICAL: Because a low density of MEF cells inhibits cell growth, care should be taken not to seed the MEFs at less than 0.7 × 10^6 cells/10 cm dish.

    k. Incubate the cells at 37°C in 5% CO_2, 95% air.
    l. Passage the cells every 2 days and scale them up to ~60–80 × 10 cm dishes.
31. Treat with MMC and prepare frozen stocks of MEFs.
    a. Dissolve 2 mg of MMC in 5 mL of the serum medium to prepare 400 μg/mL of the MMC solution.
    b. Quantify the medium volume in a dish by aspirating the medium with a 5 mL pipette and adjust the medium volume of the 10 cm dish with sub-confluent MEFs to 5 mL.
    c. Add 125 μL of the 400 μg/mL MMC solution to the culture to make the final MMC concentration of the culture medium 10 μg/mL.
    d. Incubate the cells for 2 h at 37°C in 5% CO_2, 95% air.
    e. Aspirate the medium and wash the cells with PBS three times.
    f. Aspirate PBS and add 0.25% trypsin-EDTA (1 mL/10 cm dish).
    g. Incubate the cells at 37°C in 5% CO_2 for 3 min.
    h. Tap the dish to detach the cells from the dish.
    i. Add the prewarmed serum medium (4 mL/10 cm dish), dissociate the cells by gentle pipetting, and transfer the cells into 50 mL tubes.

j. If cells are transferred into several 50 mL tubes, centrifuge the tubes at 220 × *g* for 5 min, aspirate the medium, dislodge the pellet by tapping, resuspend the cell pellet in ~0.5–1 mL of the serum medium, and combine the cells in one 50 mL tube.
k. Count the number of cells by a hemocytometer.
l. Centrifuge the cells at 220 × *g* for 3 min to pellet the cells.
m. Aspirate the supernatant and dislodge the cell pellet by gentle tapping.
n. Resuspend the cells in CELLBANKER I plus so that the cell concentration becomes ~2 × 10^6 cells/mL and transfer 500 μL to each cryopreservation tube (~1 × 10^6 cells/tube).
o. Store at −80°C (or lower temperature) and use within 12 months.

Note: In case the operation time is prolonged, the cell suspension should be kept on ice to enhance the cell viability.

MMC solution (400 μg/mL)

Reagent	Temporary concentration	Amount
Mitomycin C	400 μg /mL	2 mg
Serum Medium	n/a	~5 mL
Total	n/a	5 mL

Note: Prepare before use.

Collection of mouse embryonic testicular somatic cells.

Mouse embryonic testicular somatic cells are used for the generation of reconstituted testes (rTestes) with PGCLCs expanded on m220-5 feeder cells (steps 26–28). Prepare frozen stocks of embryonic testicular somatic cells prior to the generation of rTestes (steps 7–12) (Figure 2).

32. Isolate mouse embryonic testes.

Note: Step 32 is performed in an animal dissection room.

a. Prepare PBS, serum medium with HEPES, fine forceps, a tungsten needle, and 4-well plates.
b. Prepare pregnant female mice from a CD1 (ICR) background (specific pathogen free) at 12.5 days post coitus (dpc). We purchase them from Japan SLC, Inc.

Note: These mice typically bear ~10–12 embryos/mouse. We use 10 female mice for one experiment, which, on average, yield 100 embryonic testes and ~3–4 × 10^6 embryonic testicular somatic cells for frozen stock preparation (~3 × 10^5 cells/tube: ~10 tubes). Approximately 10–13 rTestes can be produced from one frozen tube.

c. Sacrifice pregnant female mice at 12.5 dpc by cervical dislocation.
d. Open out the abdominal cavities by surgical scissors to expose the uteri with embryo implantation, and dissect out embryonic day (E) 12.5 embryos from the uteri using fine forceps.
e. Transfer the embryos into a 10 cm dish containing ice-cold PBS to wash out the blood.
f. Transfer the embryos into a 10 cm dish containing serum medium with HEPES.
g. Dissect out the embryonic gonads with mesonephros from the abdominal parts of embryos using fine forceps.
h. Select male gonads (embryonic testes) based on their morphological appearance.
i. Bluntly excise the mesonephros with a tungsten needle (Figure 3).

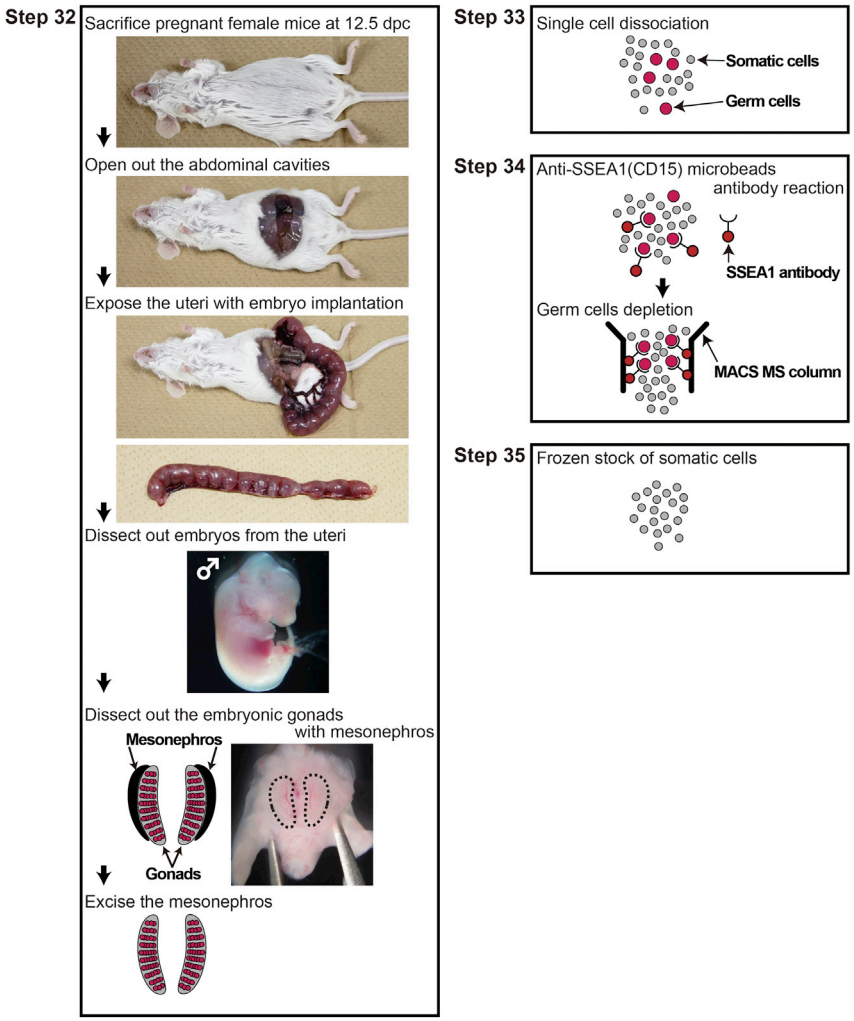

Figure 2. A schematic diagram for the collection of mouse embryonic testicular somatic cells

A schematic overview of the procedures for the collection of mouse embryonic testicular somatic cells.

Note: For information on how to prepare the tungsten needle (Figure 3), see the section "EQUIPMENT SETUP - Tungsten wire" in (Hayashi et al., 2017).

Note: The testes can be easily discriminated from the ovaries by their typical appearance with clear seminiferous tubule structures (Figure 3).

j. Transfer the testes into a well of a 4-well plate containing serum medium with HEPES.

Serum medium with HEPES		
Reagent	Final concentration	Amount
DMEM	n/a	500 mL
FBS [stock]	8.9%	50 mL
Penicillin-Streptomycin Solution [stock]	89 U/mL 89 g/mL	5 mL
GlutaMAX™-I	× 112 dilution	5 mL
HEPES	0.89 mM	500 µL
Total	n/a	560.5 mL

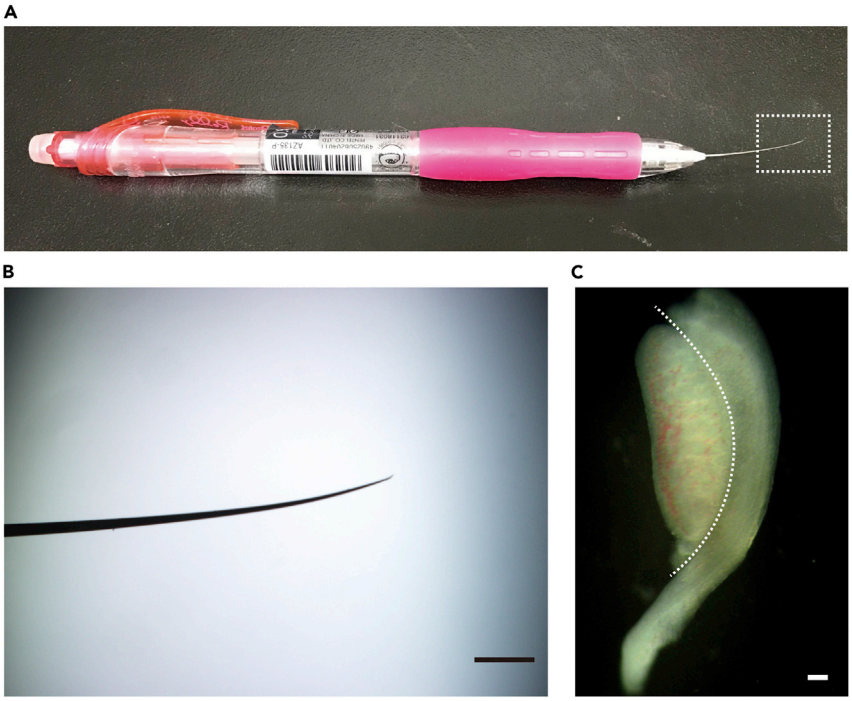

Figure 3. Isolation of embryonic testes

(A) A photomicrograph of the sharpened tungsten needle attached to a mechanical pencil.

(B) A magnified view of the boxed area in (A). Bar, 2 mm.

(C) A photomicrograph of an embryonic testis (left) with a mesonephros (right) at E12.5 (ICR background). The white dotted line indicates the border between the testis and the mesonephros to be cut with a tungsten needle. Bar, 100 μm.

Note: Store up to 1 month at 4°C.

33. Dissociate male mouse embryonic testes.
    a. Transfer the testes into a 1.5 mL tube with 1 mL of PBS.
    b. Centrifuge the tube at 200 × *g* for 1 min.
    c. Aspirate the supernatant with a P1000 pipette under a stereomicroscope to avoid accidental disposal of testes.
    d. Tap the 1.5 mL tube gently several times to loosen the testis pellet.
    e. Add 0.5 mL of 0.05% trypsin-EDTA/PBS into the 1.5 mL tube and incubate the tube at 37°C in a water bath for ~10–15 min. Tap the tube gently during the incubation.
    f. Dissociate the testes into single cells by pipetting with a P200 pipette.
    g. Add 0.5 mL of the serum medium with DNase I to inactivate trypsin and digest genomic DNA leaked out from dead cells, and pipet the cell suspension several times.
    h. Transfer the cell suspension to a 15 mL tube through a cell strainer with 70 μm pore size.
    i. Add 1 mL of the serum medium to the 1.5 mL tube and transfer it into the above 15 mL tube through the cell strainer with 70 μm pore size.
    j. Centrifuge the 15 mL tube at 200 × *g* for 5 min.
    k. Aspirate the supernatant and loosen the pellet by rigorous tapping.
34. Depletion of germ cells by magnetic cell sorting (MACS).
    a. Add 80 μL of MACS buffer to the loosened cell pellet and gently tap the tube.
    b. Add 20 μL of anti-SSEA1(CD15) microbeads into the tube.
    c. Incubate the tube on ice for 20 min. Tap gently every 10 min during the incubation.
    d. Add 5.0 mL of MACS buffer to dilute and wash the antibody.

e. Centrifuge the cell suspension at 200 × *g* for 5 min to pellet the cells.
f. Aspirate the supernatant and loosen the pellet by rigorous tapping.
g. Resuspend the cells in 1 mL of MACS buffer.
h. Set a MACS MS column onto a MiniMACS separator.
i. Apply 0.5 mL of MACS buffer to the MACS MS column to rinse it. Discard the flowthrough.

Note: To avoid non-specific cell binding and formation of air bubbles caused by drying columns, use the column immediately after rinsing.

j. Place a 15 mL tube to collect the flowthrough, which enriches embryonic testicular somatic cells.
k. Apply 1 mL of the cell suspension to the column and collect the flowthrough into the 15 mL tube.
l. Add 1 mL of MACS buffer to the column to flush out residual embryonic testicular somatic cells in the column and collect the flowthrough into the 15 mL tube. Repeat this step once.
m. Add wash buffer to the 15 mL tube to dilute the volume of the cell suspension to 10 mL and pipet well.
n. Count the number of cells using a hemocytometer.
o. Centrifuge the cell suspension at 200 × *g* for 5 min.
p. Aspirate the supernatant and loosen the pellet by rigorous tapping.

35. Prepare frozen stocks.
    a. Add CELLBANKER I plus so that the concentration of the cell suspension becomes ~5–6 × 10^5 cells/mL and transfer 500 μL to each cryopreservation tube (~2.5–3 × 10^5 cells/tube).
    b. Store at −80°C and use within 12 months.

MACS buffer

Reagent	Final concentration	Amount
PBS	n/a	15 mL
EDTA	2 mM	60 μL
7.5% BSA Fraction V	0.5%	1 mL
Total	n/a	16 mL

Note: Prepare before use.

Culture, maintenance and preparing working stock of ESCs.

For PGCLC induction, ESCs cultured in the 2iLIF medium under a feeder-free condition are used. We prepare stocks of feeder-free ESCs, 3.0–5.0 × 10^5 cells/0.5 mL of STEMCELLBANKER/tube. We recommend preparing 50–100 stock tubes.

36. Prepare poly-L-ornithine-laminin-coated wells.
    a. Typically, we use 12-well plates for ESC culture.
    b. Coat the wells with 0.5 mL of 0.01% poly-L-ornithine solution and incubate the plate at 25°C for at least 1 h.
    c. Dissolve laminin stock (~2 mg/mL) in 12 mL of PBS to prepare 300 ng/mL of laminin solution in a 15 mL tube.
    d. Wash the 0.01% poly-L-ornithine-coated wells twice with PBS.
    e. Add 1 mL/well of 300 ng/mL of the laminin solution.
    f. Incubate the plates at 37°C in 5% CO_2, 95% air for at least 1 h.

⚠ CRITICAL: The adhesiveness of ESCs to the culture wells differs substantially among the ESC lines when they are cultured in the 2iLIF medium. It is therefore critical to determine an

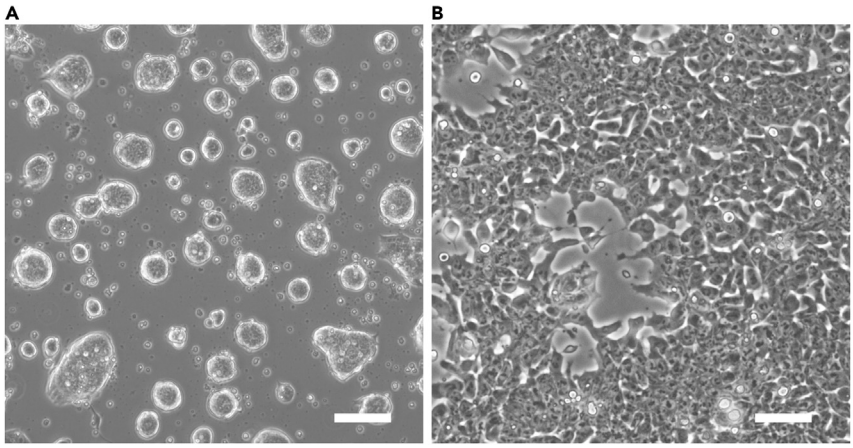

Figure 4. ESCs and EpiLCs

(A) A phase contrast image of mouse BVSCVR ESCs. Bar, 100 μm.

(B) A phase contrast image of BVSCVR EpiLCs (44 h after induction). Bar, 100 μm.

appropriate concentration of the laminin solution for each ESC line. Select the concentration with which the ESC colonies show a round, compact, and upwardly convex morphology, such as 10 ng/mL for the BVSC R8 male ESCs (Hayashi et al., 2011), or 300 ng/mL for the BVSC H18 female ESCs (Hayashi et al., 2012; Miyauchi et al., 2017).

g. Before plating ESCs, aspirate the laminin solution and wash the wells with PBS twice.
h. Add 0.5 mL/well (for a 12-well plate) of the 2iLIF medium.
i. Add the ESC suspension as prepared below.

37. Thaw frozen stocks of ESCs.
    a. Thaw frozen stocks of ESCs (~3–5 × 10^5 cells/tube) quickly by incubating the tubes in the 37°C water bath.
    b. Transfer the cells to a 15 mL tube containing more than 10 times the volume of the prewarmed wash buffer.
    c. Centrifuge the tube at 220 × *g* for 3 min.
    d. Aspirate the supernatant and loosen the pellet by rigorous tapping.
    e. Resuspend the cells in an appropriate volume (~1 mL/tube) of the 2iLIF medium.
    f. Transfer the ESC suspension to the wells prepared in step 36 (typically ES cells/tube/well for a 12-well plate).
    g. Incubate the cells at 37°C in 5% CO_2, 95% air for ~24–48 h.
    h. If the cells are confluent (Figure 4A), passage the cells as in step 38.
38. Passage ESCs.
    a. Prepare poly-L-ornithine-laminin-coated wells (step 36), wash the wells twice with PBS, and add 1 mL/well of 2iLIF.
    b. Typically, ESCs become confluent (~1.0–1.5 × 10^6 cells/well of a 12-well plate) after 48 h of culture (we start from 1.0–2.0 × 10^5 cells/well of a 12-well plate).
    c. Aspirate the medium from the well for a 12-well plate with confluent ESCs.
    d. Add 0.5 mL of prewarmed TrypLE Express/well for a 12-well plate.
    e. Incubate the plate at 37°C in 5% CO_2, 95% air for 4 min.
    f. Dissociate the cells into single cells by pipetting several times and transfer the cells into a 15 mL tube.
    g. Add 2 mL/well of the prewarmed wash buffer, and transfer the buffer to the 15 mL tube.
    h. Count the number of cells using a hemocytometer.

i. Centrifuge the 15 mL tube at 220 × *g* for 3 min and aspirate the supernatant.
j. Resuspend the cells in 1 mL/well of the 2iLIF medium so that the concentration of the cell suspension becomes about ~1.0–1.5 × 10^6 cells/mL.
k. Transfer 0.1 mL of the cell suspension (~1.0–1.5 × 10^5 cells) to each well.
l. Incubate the cells at 37°C in 5% CO_2, 95% air.

39. Scale up and prepare working stocks of ESCs.
    a. Prepare poly-L-ornithine-laminin-coated wells for a 6-well plate (step 36), wash the wells twice with PBS, and add 3 mL/well of 2iLIF.
    b. Transfer ~0.2–0.3 mL of the ESC suspension (~3.0–4.0 × 10^5 cells) into the wells of the poly-L-ornithine-laminin-coated 6-well plates.
    c. Incubate the cells at 37°C in 5% CO_2, 95% air for 48 h.

    Note: Typically, ESCs become confluent (~2.0–2.5 × 10^6 cells/well of a 6-well plate) after 48 h of culture. From one 6-well plate, ~1.5 × 10^7 ESCs are harvested, and ~50 working-stock tubes (3 × 10^5 cells/tube) are prepared.

    d. Aspirate the medium, and add 1 mL of prewarmed TrypLE Express/well of a 6-well plate.
    e. Incubate the plate at 37°C in 5% CO_2, 95% air for 4 min.
    f. Dissociate the cells into single cells by pipetting several times and transfer the cells into a 50 mL tube.
    g. Add 4 mL/well of the prewarmed wash buffer, wash the wells thoroughly, and transfer the buffer to the 50 mL tube.
    h. Count the number of cells using a hemocytometer.
    i. Centrifuge the 50 mL tube at 220 × *g* for 3 min and aspirate the supernatant.
    j. Add STEM-CELLBANKER1 so that the concentration of the cell suspension becomes ~6.0–10 × 10^5 cells/mL and transfer 500 μL to each cryopreservation tube (3.0–5.0 × 10^5 cells/tube).
    k. Store at −80°C.

2iLIF medium

Reagent	Final concentration	Amount
N2B27	×1	40 mL
CHIR99021	3 μM	4.0 μL
PD0325901	0.4 μM	1.6 μL
LIF	10^3 U/mL	4.0 μL
Total	n/a	40 mL

Note: Store up to 2 weeks at 4°C. At the time of use, dispense only the volume to be used and keep it warm at 37°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
0.25% Trypsin-EDTA (Phenol Red Plus)	Thermo Fisher Scientific	25200056
0.5% Trypsin-EDTA (Phenol Red Free)	Thermo Fisher Scientific	15400054
2-Mercaptoethanol	Thermo Fisher Scientific	21985023
5 M EDTA	Nacalai	06894-14
Activin A (Human/Mouse/Rat)	PeproTech	120-14
AlbuMaxI	Gibco	11020062
Anti-SSEA1(CD15) Microbeads	Miltenyi Biotec	#130094530

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Apo Transferrin	Sigma	T1147
B27	Thermo Fisher Scientific	12587010
Bone morphogenetic protein-4 (BMP4), Human, Recombinant	R&D	#314-BP
BSA Fraction V	Gibco	15260-037
CELLBANKER 1plus	Nippon Zenyaku	130-042-201
CHIR99021	BioVision	4423
Cyclosporin A	Sigma	30024
DMEM/F12	Gibco	11330-057
DMEM/F12 (HEPES and Phenol Red Free)	Thermo Fisher Scientific	21041025
DNase I	Sigma	D5025
Dulbecco's Modified Eagle Medium (DMEM)	Gibco	10313-021
EGF, Mouse, Recombinant, Carrier-free	R&D	2028EG
Fetal Bovine Serum (FBS)	HyClone	SH30910.03
FGF2	Invitrogen	13256029
Fibronectin (Human)	Merck Millipore	FC010
Forskolin	Sigma	F3917
GDNF (glial cell line-derived neurotrophic factor), Rat, Recombinant	R&D	512GF
Gelatin from bovine skin	Sigma-Aldrich	G9391
Glasgow's MEM (GMEM)	Thermo Fisher Scientific	11710035
GlutaMAX	Life Technologies	35050061
HEPES (1 M)	Thermo Fisher Scientific	15630106
Insulin (powder)	Sigma	#I-1882, Lot # SLBN0545V
Insulin-Transferrin-Selenium (ITS)-G	Gibco	41400045
KnockOut™ Serum Replacement (KSR)	Gibco	10828028
Laminin	BD Biosciences	354232
L-Glutamine	Life Technologies	25030149
LIF (ESGRO®)	Merck Millipore	ESG1107
MEM Non-Essential Amino Acids Solution	Thermo Fisher Scientific	11140-050
MEM α (GlutaMAX™, No Nucleosides)	Gibco	32561-037
Minimum Essential Medium (MEM) Vitamin Solution	Thermo Fisher Scientific	11120052
Mitomycin C (MMC)	Sigma-Aldrich	M4287
Neurolbasal™ Medium	Invitrogen	2113-049
PBS	Thermo Fisher Scientific	10010023
PD325901	Stemgent	04-0006
Penicillin-Streptomycin (10^4 units/mL, 10 mg/mL)	Life Technologies	15140148
Poly-L-Ornithine	Sigma	P3655
Progesterone	Sigma	P8783
Putrescine	Sigma	P5780
Recombinant Human BMP-4	R&D	314BP01M
Recombinant Mouse SCF	R&D	455MC
Rolipram	Abcam	AB120029
Sodium Pyruvate	Thermo Fisher Scientific	11360-070
Sodium Selenate	Sigma	S5261
STEM-CELLBANKER (GMP grade)	Nippon Zenyaku	CB047
Stem cell factor (SCF), Mouse, Recombinant	R&D	#455-MC
StemPro™-34 SFM (1×)	Gibco	10639011
TrypLE Express	Thermo Fisher Scientific	12604-021
Experimental models: Cell lines		
BVSCVR-ESC (Blimp1-mVenusTg/0; Stella-ECFPTg/0; Ddx4/Mvh-RFPTg/0, B6C3(Cg) ×C57BL/6, XY)	(Miyauchi et al., 2017)	n/a

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
m220-5	(Ohta et al., 2017)	n/a
Experimental models: Organisms/strains		
Mouse/Slc:ICR, pregnant female mice at 12.5 days post coitus (dpc)	Japan SLC, Inc.	n/a
Other		
10 cm Dish	Falcon	353003
12-Well Plate	Falcon	353043
15 cm Dish	Falcon	353025
24-Well Plate	Falcon	353047
4-Well Plate	Thermo Fisher Scientific	176740
50 mL Tube	IWAKI	2345-050
6-Well Plate	Falcon	353046
96-Well Flat Plate	Falcon	353072
Cell Strainer with 35 μm Pore Size (5 mL round-bottom polystyrene test tubes)	Falcon	352235
Cell Strainer with 70 μm Pore Size (pluriStrainer-Mini 70 μm)	pluriSelect	43-10070-60
Centrifuge	Eppendorf	Centrifuge 5702
CO^2 Incubator	SANYO	MCO-18AIC
Cryotube	Thermo Fisher Scientific	375418
Disposable Sterile Plastic Pipettes (5, 10, 25 and 50 mL)	Falcon	n/a
Fine Forceps	FST	#11252-30
FACS Aria III	BD	n/a
FlowJo™	BD	v10.7.1
Hemocytometer	WATSON	177-112C
MS Column	Miltenyi Biotec	130-042-201
MiniMACS Separator	Miltenyi Biotec	130-090-312
Nunclon Sphera 96-Well Microplate	Nunc	174925
Surgical Scissors	Natsume Seisakusho	B-12-H
TOMY-CAX370 Centrifuge	TOMY Industries, Ltd.	n/a

MATERIALS AND EQUIPMENT

Fibronectin solution		
Reagent	Final concentration	Amount
Fibronectin [stock]	16.7 μg/mL	10 μL
PBS	n/a	600 μL
Total	n/a	610 μL

Note: Prepare before use.

EpiLC induction medium		
Reagent	Final concentration	Amount
N2B27	n/a	1 mL
KnockOut™ Serum Replacement (KSR)	1%	10 μL
bFGF	12 ng/mL	1.2 μL
Activin A	20 ng/mL	0.4 μL
Total	n/a	1 mL

Note: Prepare before use and prewarm at 37°C.

PGCLC induction medium		
Reagent	Final concentration	Amount
GMEM	n/a	8.1 mL
2-Mercaptoethanol	0.1 mM	18 μL
KnockOut™ Serum Replacement (KSR)	15%	1.5 mL
Penicillin-Streptomycin Solution	100 U/mL 100 g/mL	100 μL
L-Glutamine Solution	2 mM	100 μL
Non-Essential Amino Acids Solution	0.1 mM	100 μL
Sodium Pyruvate	1 mM	100 μL
BMP4	500 ng/mL	100 μL
Stem Cell Factor (SCF)	100 ng/mL	20 μL
EGF	50 ng/mL	1 μL
LIF	10^3 U/mL	1 μL
Total	n/a	10 mL

Note: Prepare before use and prewarm at 37°C.

PGCLC culture medium		
Reagent	Final concentration	Amount
GMEM	n/a	8.35 mL
2-Mercaptoethanol	0.1 mM	18 μL
FBS	2.5%	0.25 mL
KnockOut™ Serum Replacement (KSR)	10%	1.0 mL
Penicillin-Streptomycin Solution	100 U/mL 100 g/mL	100 μL
L-Glutamine Solution	2 mM	100 μL
Non-Essential Amino Acids Solution	0.1 mM	100 μL
Sodium Pyruvate	1 mM	100 μL
Stem Cell Factor (SCF)	100 ng/mL	20 μL
Forskolin	10 μM	3.3 μL
Rolipram	10 μM	2.0 μL
Cyclosporine A	5 μM	1.0 μL
Total	n/a	10 mL

Note: Prepare before use and prewarm at 37°C.

rTestis floating culture medium		
Reagent	Final concentration	Amount
GMEM	n/a	8.35 mL
2-Mercaptoethanol	0.1 mM	18 μL
FBS	2.5%	0.25 mL
KnockOut™ Serum Replacement (KSR)	10%	1.0 mL
Penicillin-Streptomycin Solution	100 U/mL 100 g/mL	100 μL
L-Glutamine Solution	2 mM	100 μL
Non-Essential Amino Acids Solution	0.1 mM	100 μL
Sodium Pyruvate	1 mM	100 μL
Stem Cell Factor (SCF)	100 ng/mL	20 μL
Forskolin	10 μM	3.3 μL
Rolipram	10 μM	2.0 μL
Total	n/a	10 mL

Note: Prepare before use and prewarm at 37°C.

rTestis culture medium		
Reagent	Final concentration	Amount
MEM α	n/a	35.2 mL
2-Mercaptoethanol	0.1 mM	72 μL
Penicillin-Streptomycin Solution	100 U/mL 100 g/mL	400 μL
GlutaMAX™-I	× 100 dilution	400 μL
FBS	10%	4.0 mL
Total	n/a	40 mL

Note: Prepare before use.

GSC culture medium STOCK		
Reagent	Final concentration	Amount
StemPro34 with StemPro supplement	n/a	465 mL
2-Mercaptoethanol	0.05 mM	454 μL
FBS [stock]	1%	5 mL
MEM Vitamin Solution	× 100 dilution	5 mL
AlbuMAX® I	5.0 mg/mL	2.5 g
Penicillin-Streptomycin Solution [stock]	100 U/mL 100 g/mL	5 mL
Non-Essential Amino Acids Solution [stock]	0.1 mM	5 mL
Sodium Pyruvate [stock]	1 mM	5 mL
GlutaMAX™-I	× 100 dilution	5 mL
ITS-G	× 100 dilution	5 mL
Total	n/a	500 mL

Note: Dispense 40 mL of the mixture into 50 mL tubes and store at −80°C. Once thawed, store at 4°C and use within 2 weeks.

GSC culture medium		
Reagent	Final concentration	Amount
GSC Culture Medium STOCK	n/a	40 mL
GDNF [stock]	20 ng/mL	40 μL
bFGF [stock]	10 ng/mL	40 μL
LIF [stock]	10^3 U/mL	4 μL
EGF [stock]	20 ng/mL	1.6 μL
Total	n/a	40 mL

Note: After cytokines are added, store the medium for up to 2 weeks at 4°C.

STEP-BY-STEP METHOD DETAILS

Induction of epiblast-like cells (EpiLCs) from mESCs

Timing: 2 days for steps 1–3

This section accomplishes the induction of EpiLCs from mESCs.

1. Prepare fibronectin-coated wells for EpiLC induction.
   a. Coat the wells with 610 μL/well of fibronectin solution.
   b. Incubate the plate at 37°C in 5% CO_2, 95% air for at least 1 h.
2. Induce EpiLCs.

a. Prepare ESCs (in the logarithmically growing phase) as in step 38 in the "BEFORE YOU BEGIN" section.

Note: Typically, we use thawed ESCs for EpiLC induction after two or three passages.

b. Aspirate the 2iLIF medium for ESCs cultured in a 12-well plate.
c. Add 0.5 mL/well (for a 12-well plate) of prewarmed TrypLE Express.
d. Incubate the plate at 37°C in 5% CO_2, 95% air for 4 min.
e. Dissociate ESCs into single cells by pipetting them several times.
f. Transfer the cells into a 15 mL tube.
g. Add 2 mL/well (for a 12-well plate) of the prewarmed wash buffer, wash the well thoroughly, and transfer the buffer to the 15 mL tube.
h. Centrifuge the tube at 220 × *g* for 3 min to pellet the cells.
i. Aspirate the supernatant and dislodge the cells by tapping.
j. Resuspend the ESCs in 1 mL of EpiLC induction medium.
k. Count the number of cells by a hemocytometer.
l. Aspirate the fibronectin solution from the fibronectin-coated 12-well plate prepared in step 1.
m. Immediately add 1 mL/well of the prewarmed EpiLC induction medium.
n. Transfer 1.0 × 10^5 cells of the ES cell suspension in EpiLC induction medium to a well of the fibronectin-coated 12-well plate.
o. Incubate the plate at 37°C in 5% CO_2, 95% air for 24 h.

⚠ CRITICAL: The KSR concentration (1%) of the EpiLC induction medium is critical for PGCLC induction. A KSR concentration higher than 1% results in inefficient PGCLC induction.

3. Change the EpiLC induction medium.
   a. Aspirate the medium and add 1 mL of pre-warmed fresh EpiLC induction medium.
   b. Incubate the plate at 37°C in 5% CO_2, 95% air for 20 h (Figure 4B).

⚠ CRITICAL: During ESC-to-EpiLC induction, EpiLCs acquire the competence to differentiate into PGCLCs in a transient manner, and thus it is critical to determine the appropriate induction time for each ESC line (usually around 48 h after EpiLC induction). Start with 0.8–1 × 10^5 ESCs/well of a 12-well plate and determine the time point when they grow to 1 × 10^6 cells/well (a sub-confluent state, Figure 4B), which is often the best time for PGCLC induction. Overgrowth of EpiLCs leads to an impaired PGCLC induction efficiency. The BVSCVR ESCs show a robust PGCLC differentiation potential at 44 h after induction into EpiLCs.

Induction of PGCLCs from EpiLCs

Timing: 4 days for step 4

This section accomplishes the induction of PGCLCs from mEpiLCs.

4. PGCLC induction.
   a. Initiate PGCLC induction at 44 h after induction into EpiLCs (Figure 4B).
   b. Aspirate the medium and wash the wells of EpiLCs with 1 mL/well (for a 12-well plate) of PBS.
   c. Add 0.5 mL/well (for a 12-well plate) of prewarmed TrypLE Express.
   d. Incubate the plate for 2 min at 25°C.
   e. Add 2 mL/well (for a 12-well plate) of prewarmed wash buffer and dissociate EpiLCs into single cells by an appropriate pipetting.
   f. Transfer the cells into a 15 mL tube.

g. Centrifuge the tube at 220 × *g* for 3 min to pellet the cells.
h. Aspirate the supernatant and dislodge the cells by tapping.
i. Resuspend the cells with the PGCLC induction medium so that the cell concentration becomes 4,000 cells/100 μL (4×10^4 cells/mL).

⚠ CRITICAL: For the BVSCVR line, a concentration of 4,000 cells/100 μL leads to a good PGCLC induction efficiency. Determine the appropriate cell concentration for each line (typically, 2,000 cells/100 μL (2×10^4 cells/mL) –5,000 cells/100 μL (5×10^5 cells/mL)).

j. Transfer 4,000 cells/100 μL of the EpiLC suspensions into single wells of Nunclon Sphera 96-well Microplates.
k. Incubate the plates for ~90–96 h at 37°C in 5% CO_2, 95% air.

⚠ CRITICAL: The expansion potential of PGCLCs upon PGCLC culture depends on the induction time of PGCLCs and thus it is critical to determine an appropriate PGCLC induction time for each ESC line. The BVSCVR ESC-derived PGCLCs show an appropriate expansion potential after ~90 h of PGCLC induction [after ~4 days of PGCLC induction: d4 PGCLCs], at which time PGCLCs show a robust BV fluorescence, but have only just initiated SC fluorescence (see below (Figure 5)). Fully BV^+ and SC^+ PGCLCs show a low expansion potential.

PGCLC expansion culture

Timing: 5 days for steps 5–7

This section accomplishes the expansion of PGCLCs.

5. Prepare culture plates with m220-5 feeders.
   a. Coat the wells with 0.5 mL/well (for a 24-well plate) of 0.1% gelatin solution.
   b. Incubate the plate for 30 min at 37°C in 5% CO_2, 95% air.
   c. Thaw the m220-5 frozen stock tube (~$4–6 \times 10^6$ cells in 500 μL/tube) quickly by incubating the tubes in the 37°C water bath.
   d. Transfer the cells to a 15 mL tube containing more than 10 times the volume of the prewarmed wash buffer.
   e. Centrifuge the tube at 220 × *g* for 3 min.
   f. Aspirate the supernatant and dislodge the pellet by tapping.
   g. Resuspend the cells with serum medium so that the cell concentration becomes 1×10^6 cells/mL.
   h. Aspirate the gelatin solution of the wells, and transfer ~$2.5–5 \times 10^5$ cells/0.5 mL/well of the m220-5 cell suspension to one well of a 24-well plate.

⚠ CRITICAL: the density of m220-5 feeder cells is critical for PGCLC expansion. Low density of m220-5 feeder cells leads to low PGCLC expansion efficiency.

   i. Incubate the plate at 37°C in 5% CO_2, 95% air for more than 4 h until the feeder cells attach over the entire surface of the wells.
   j. After the attachment of cells, replace the medium with 1 mL/well of the PGCLC culture medium.
6. Sort BV^+ PGCLCs (d4 PGCLC) by fluorescence-activated cell sorting (FACS).
   a. Add 5 μL of DNase I stock in 1 mL of serum medium to prepare serum medium with DNase I.

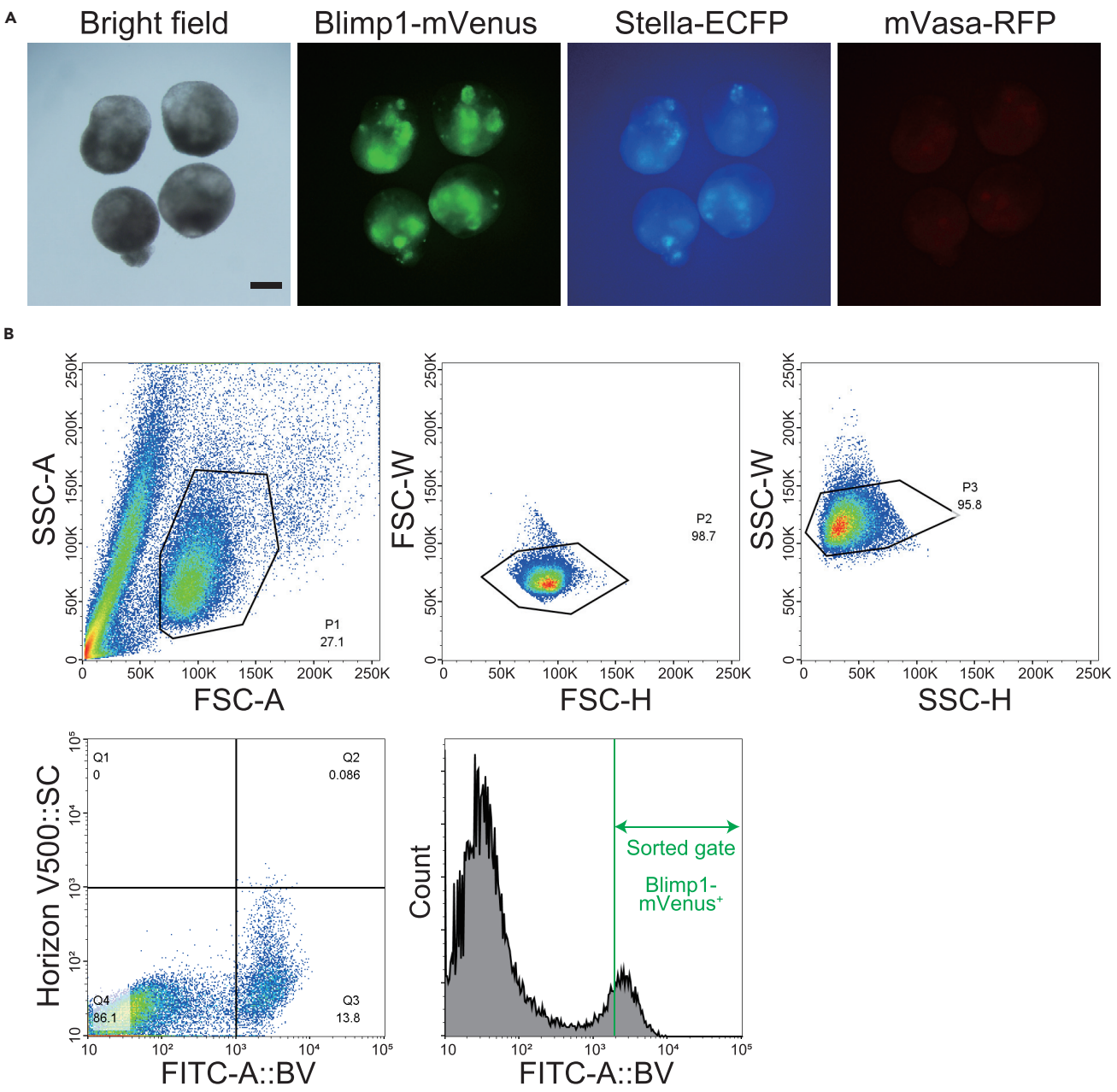

Figure 5. Induction of d4 PGCLCs

(A) Bright field (left) and fluorescent (second left: Blimp1-mVenus; second right: Stella-ECFP; right: mVasa-RFP) images of EpiLC aggregates (90 h after induction) containing d4 PGCLCs observed with a fluorescence stereomicroscope. Bar, 100 μm.

(B) A flow cytometric gating strategy for sorting BV^+ d4 PGCLCs. The P1 gate indicates depletion of dead cells (x-axis: FSC-A; y-axis: SSC-A) (upper left). The P2 gate is for single cell selection (x-axis: FSC-H; y-axis: FSC-W) (upper middle). The P3 gate is for single cell selection (x-axis: SSC-H; y-axis: SSC-W) (upper right). BVSC sorting is also shown (x-axis: BV; y-axis: SC) (lower left). A histogram of the P3 population. The gate for the BV^+ cell sorting is indicated (lower right).

b. Add 5 μL of DNase I stock in 1 mL of wash buffer to prepare wash buffer with DNase I.

c. Collect the d4PGCLC aggregates after ~90–96 h of PGCLC induction into a 1.5 mL tube containing 1 mL of the PBS. We collect the aggregates by using a P200 pipette under a stereomicroscope.

d. Centrifuge the tube at 220 × *g* for 1 min.
e. Remove the supernatant by using a P1000 pipette under a stereomicroscope to avoid accidental disposal of aggregates.
f. Add 0.5 mL/tube of prewarmed 0.05% trypsin-EDTA/PBS and tap the tube gently several times.
g. Incubate the tube in the 37°C water bath for 10 min.
h. Periodically, tap the tube to ensure that the aggregate is fully exposed to the 0.05% trypsin-EDTA/PBS solution.
i. Dissociate the aggregates into single cells by carefully and rigorously pipetting with a P200 pipette.
j. Add 0.5 mL (in a volume equal to that of the 0.05% trypsin-EDTA-PBS) of serum medium with DNase I and pipet the suspension several times to complete the dissociation under a stereomicroscope.

⚠ CRITICAL: The aggregates should be dissociated into single cells as much as possible. Sticky cell masses caused by genomic DNAs leaking from dead cells should be disaggregated by DNase I treatment. Insoluble aggregates caused by genomic DNAs significantly reduce the efficiency for PGCLC sorting.

k. Centrifuge the tube at 200 × *g* for 5 min.
l. Aspirate the supernatant and dislodge the pellet by rigorous tapping.
m. Resuspend the pellet in the wash buffer with DNase I so that the cell concentration becomes about 5 × 10^6/mL.
n. Transfer the cell suspension through a cell strainer with 35 μm pore size to a 5 mL tube.
o. Keep the tube on ice until application to FACS.
p. Sort the BV^+ PGCLCs (d4 PGCLC) using a flow cytometer according to the manufacturer's instructions. We use FACS Aria III (BD Bioscience).
q. Use a 15 mL tube containing 10 mL of wash buffer to collect the sorted cells.
r. Record the number of sorted cells as indicated in the "sort layout" for step 7.

⚠ CRITICAL: To obtain a high-purity cell fraction, we select the "Purity" setting of the "Sort Precision Mode". To maintain the viability of sorted cells, we cooled the collection tube holder by using the water bath system of the BD Temperature Control Option.

⚠ CRITICAL: See Figure 5B (lower right) for an appropriate gating of the BV^+ PGCLCs (d4 PGCLC) fraction: perform a stringent gating for BV positivity to exclude the contamination of BV-negative cells as much as possible. We typically sort BV-positive cells with BV signal intensity higher than one-third of the BV-positive population.

7. PGCLC expansion culture.
a. Centrifuge the 15 mL tube containing d4 PGCLCs at 220 × *g* for 5 min.
b. Aspirate the supernatant and dislodge the cells by tapping.
c. Resuspend d4 PGCLCs in the PGCLC culture medium so that the cell concentration becomes ~1.0 × 10^4/mL (refer to step 6-r for the number of cells).
d. Transfer 500 μL of the d4PGCLC suspension (5 × 10^3 cells) into a well of the 24-well plate with m220-5 feeder cells (step 5).
e. Incubate the plate for 120 h at 37°C in 5% CO_2, 95% air.
f. Change half the volume of the culture medium at culture days 1 and 3.

⚠ CRITICAL: In addition to m220 feeder cells, d4 PGCLCs should also be cultured at appropriate densities. Low densities of these cells might lead to a low expansion rate of d4 PGCLCs.

Generation of reconstituted testes (rTestes)

Timing: 2 weeks for steps 8–13

This section accomplishes the generation of rTestes and their culture for differentiating PGCLCs into spermatogonium-like cells.

8. Sort culture day 5 BV+SC+ PGCLCs (d4c5 PGCLCs) (Figure 6A) and prepare the d4c5 PGCLCs suspension.
   a. Add 5 μL of DNase I stock per 1 mL of wash buffer to prepare the wash buffer with DNase I.
   b. Wash the wells of the 24-well plate containing the d4c5 PGCLCs with 1 mL/well of PBS.
   c. Add 0.5 mL/well of prewarmed TryPLE Express and incubate the plate at 37°C for 4 min.
   d. Add 1 mL/well of prewarmed wash buffer with DNase I and dissociate the cells into single cells by pipetting them several times with a P1000 pipette.
   e. Transfer the cell suspension into a 15 mL tube and add 1 mL of wash buffer (making the total volume 2.5 mL, i.e., a 5-fold dilution of the TryPLE Express).
   f. Centrifuge the tube at 200 × *g* for 5 min.
   g. Aspirate the supernatant and dislodge the pellet by rigorous tapping.
   h. Resuspend the cells in wash buffer with DNase I so that the cell concentration becomes ~5 × 10^6/mL.
   i. Transfer the cell suspension through a cell strainer with 35 μm pore size to a 5 mL tube.
   j. Keep the tube on ice until application to FACS.
   k. Sort the BV^+SC^+ PGCLCs (d4c5 PGCLCs) using a flow cytometer according to the manufacturer's instructions. We use FACS Aria III (BD Bioscience) (Figure 6B).
   l. Use a 15 mL tube containing 10 mL of wash buffer to collect the sorted cells.
   m. Record the number of sorted cells as indicated in the "sort layout" for step 8-p.

   ⚠ CRITICAL: To obtain a high-purity cell fraction, we select the "Purity" setting of the "Sort Precision Mode". To maintain the sorted cell viability, we cool the collection tube holder by using the water bath system of the BD Temperature Control Option.

   n. Centrifuge the 15 mL tube with sorted cells at 220 × *g* for 5 min.
   o. Aspirate the supernatant, dislodge the cells by tapping, and resuspend the cells in the rTestis floating culture medium so that the cell concentration becomes ~1.0 × 10^4 cells/100 μL (~1.0 × 10^5 cells/mL) (refer to step 8-n for the number of cells).
   p. Keep the tube on ice.
9. Prepare the embryonic testicular somatic cell suspension.
   a. Thaw the frozen stocks of mouse embryonic testicular somatic cells (~2.5–3 × 10^5 cells/tube) quickly by incubating the tubes in the 37°C water bath.
   b. Transfer the cells into a 15 mL tube containing more than 10 times the volume of the prewarmed wash buffer.
   c. Centrifuge the tube at 220 × *g* for 5 min.
   d. Aspirate the supernatant and resuspend the cells in 500 μL of rTestis floating culture medium.
   e. Count the number of cells by a hemocytometer.
   f. Add an appropriate volume of rTestis floating culture medium so that the cell concentration becomes ~2.0 × 10^4 cells/100 μL (2.0 × 10^5 cells/mL).
   g. Keep the tube on ice.
10. Prepare floating aggregates of rTestes.
   a. Mix an equal volume of the d4c5 PGCLCs suspension and the embryonic testicular somatic cell suspension.
   b. Transfer 200 μL of the mixed cell suspension to each well of a low binding 96-well plate so that each rTestis consists of 1.0 × 10^4 d4c5 PGCLCs and 2.0 × 10^4 embryonic testicular somatic cells.

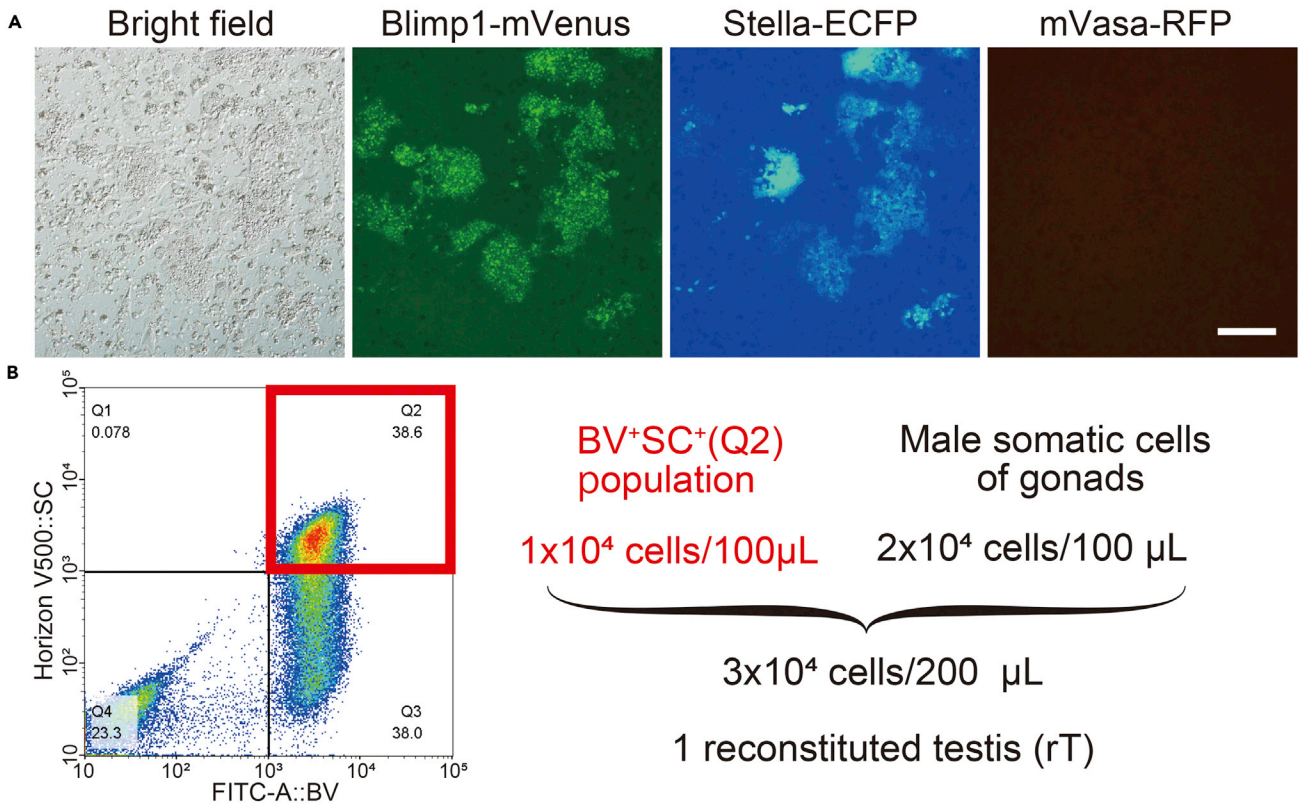

Figure 6. Purification of d4c5 PGCLCs

(A) Bright field (left) and fluorescent (second left: BV; second right: SC; right: VR) images of d4c5PGCLCs. Bar, 100 μm.

(B) A flow cytometric gating strategy for sorting BV^+SC^+ d4c5 PGCLCs. The Q2 gate for the BV^+SC^+ d4c5 PGCLCs is indicated (left). One rTestis is composed of 1 × 10^4 cells (BV^+SC^+ d4c5 cells) and 2 × 10^4 cells (male somatic cells of gonads) (right).

c. Incubate the plate for 48 h at 37°C in 5% CO_2, 95% air to generate floating aggregates of rTestes.

11. Prepare rTestes culture plates for air–liquid interphase culture.
    a. Add 1 mL of distilled water to the peripheral 16 wells of the 24-well plates to maintain the humidity of the culture.
    b. Add 700 μL/well of the rTestis culture medium to the inner 8 wells of the plates.
    c. Set a culture membrane insert (ThinCert™) into a well with the rTestis culture medium.

Note: Culture membrane inserts should be immersed in the medium. Avoid air-bubble formation between the membrane inserts and the medium.

Note: For rTestes culture under an air–liquid interphase condition, we use ThinCert™ (24-well plates and PET membrane inserts with 1 μm pore size and 2 × 10^6/cm^2 pore density).

12. Transfer rTestes onto the membrane inserts.
    a. Transfer an rTestis cultured under a floating aggregate condition for 48 h in step 10 onto the center of a membrane insert using a glass capillary equipped with a mouth pipette (Figures 7A and 7B (left)). Place one rTestis per one membrane insert.
    b. Aspirate the medium carried over around the rTestis on the membrane using a thin capillary (Figures 7A and 7B (right)) by capillary reaction.

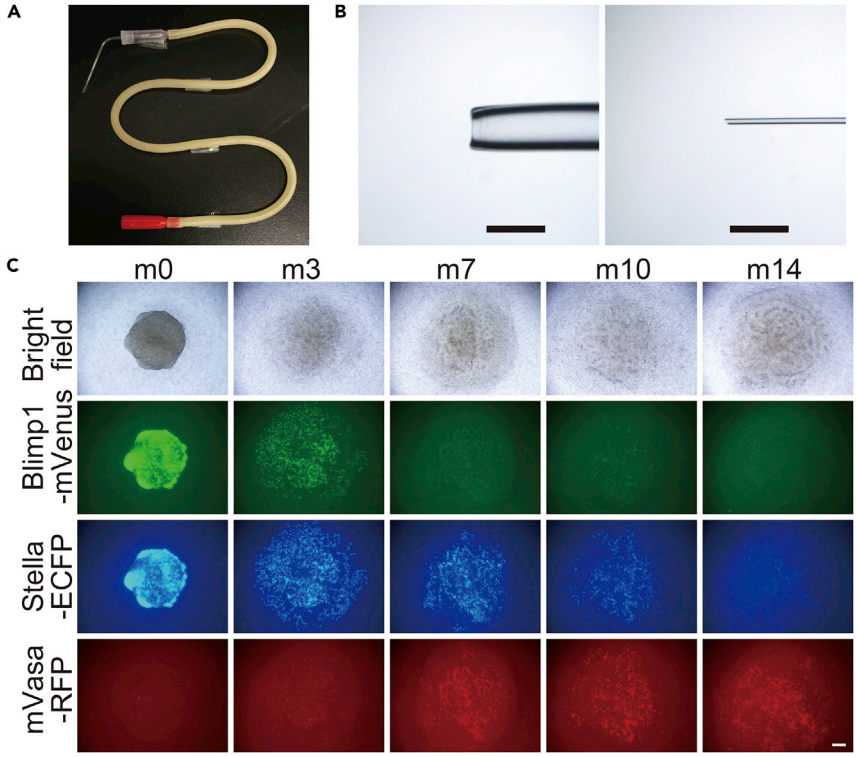

Figure 7. Generation and culture of rTestes

(A) A photomicrograph of a mouth pipette equipped with a glass capillary.

(B) Magnified images of the tips of the glass capillaries for transferring the rTestes onto the membrane inserts (left) or for aspirating extra culture fluid (right). Bar, 2 mm.

(C) Bright field (left) and fluorescent (second left: BV; second right:SC; right: VR) images of the m0 (floating d2) and the m3, m7, m10, m14 rTestis under the air–liquid interface culture. Bar, 100 μm.

⚠ CRITICAL: Transfer rTestes onto the membranes with as little medium carryover as possible.

13. Culture rTestes under an air–liquid interface condition (Figure 7C).
    a. Culture the rTestes at 37°C in 5% CO_2, 95% air for 2 weeks.
    b. Replace half the medium with fresh rTestes culture medium every 2 days.

Note: We define the starting day of rTestis culture on the membrane as membrane culture day (m0).

⚠ CRITICAL: Examine the rTestis culture periodically to evaluate whether the rTestes are undergoing appropriate development. See (Ishikura et al., 2021) for indexes (rTestis morphology and the timing of marker-gene expression) of the appropriate development of rTestes.

⚠ CRITICAL: During the culture, the medium may penetrate the membranes and submerge rTestes. In such a case, place a P200 pipette against the surface of the membranes and gently absorb the culture medium on the membranes.

GSCLC (germline stem-cell-like cells) derivation

Timing: 1.5 months for steps 14–21

This section accomplishes the derivation of GSCLCs from the PGCLC-derived spermatogonium-like cells in rTestes cultured for 14 days according to the GSC derivation procedures (Kanatsu-Shinohara et al., 2003) with some modification.

14. Prepare culture plates with MEFs for GSCLC derivation.
    a. Coat the wells of a 96-well plate (flat-bottom) with 0.1 mL/well of 0.1% gelatin solution and incubate the plate for 30 min at 37°C in 5% CO_2, 95% air.
    b. Thaw the MEF frozen stock tube (1×10^6 cells/tube) quickly by incubating the tubes in the 37°C water bath.
    c. Transfer the cells into a 15 mL tube containing more than 10 times the volume of the pre-warmed wash buffer.
    d. Centrifuge at 220 × *g* for 3 min.
    e. Aspirate the supernatant and dislodge the pellet by tapping.
    f. Resuspend the cells with an appropriate volume of serum medium so that the cell concentration becomes ~3.0×10^5 cells/mL.
    g. Aspirate the gelatin solutions of the 96-well flat plate.
    h. Transfer 100 μL of the MEF suspension to the wells (3.0×10^4 cells/well).
    i. Incubate the plate at 37°C in 5% CO_2, 95% air for more than 4 h until the MEFs attach over the entire surface of the wells.
    j. After attachment of the MEFs, replace the medium with 100 μL/well of the GSC culture medium.

⚠ CRITICAL: for GSCLC derivation from PGCLC-derived spermatogonium-like cells, it is critical to seed the wells with a high concentration of MEFs (e.g., ~$3.0–5.0 \times 10^4$ cells/well for a 96-well plate).

15. Collection of rTestes from the culture inserts.
    a. Add 1 mL of 0.5% trypsin-EDTA per 1 mL of PBS to prepare 0.25% trypsin-EDTA-PBS.
    b. Transfer a culture insert with an rTestis to a well of a new 24-well plate without medium.

    Note: Perform this quickly to prevent the rTestes from drying out.

    c. Add 0.3 mL/well of PBS to the wells to wash the culture insert and aspirate PBS.
    d. Add 0.3 mL/well of prewarmed 0.25% trypsin-EDTA-PBS to the wells.
    e. Scrape off an rTestes from a culture insert using a P1000 pipette under a stereomicroscope.
    f. Transfer an rTestis into a 1.5 mL tube with 0.3 mL/tube of 0.25% trypsin-EDTA-PBS.
16. Prepare to sort the PGCLC-derived spermatogonium-like cells.
    a. Add 5 μL of DNase I stock per 1 mL of serum medium to prepare the serum medium with DNase I.
    b. Add 13.3 μL of 7.5% BSA Fraction V per 1 mL of PBS to prepare the antibody buffer.
    c. Incubate the 1.5 mL tube containing an rTestis (step 15-f) in the 37°C water bath for ~10–15 min with periodical tapping.
    d. Dissociate the rTestis into single cells by rigorous pipetting with a P200 pipette. Perform the dissociation under a stereomicroscope for visual inspection of the degree of dissociation.
    e. Add 0.6 mL of the serum medium with DNase I.
    f. Centrifuge the tube at 200 × *g* for 5 min.
    g. Aspirate the supernatant and dislodge the pellet by tapping.
    h. Resuspend the cells with 50 μL of antibody buffer.
    i. Add 5 μL of anti-CD9 antibody conjugated with APC and mix the cell suspension well.

    Note: To strictly distinguish the autofluorescence from the VR reporter fluorescence of the spermatogonium-like cells, we use anti-CD9 antibody conjugated with APC. MCAM

(Kanatsu-Shinohara et al., 2012) can also be used as a surface marker for spermatogonium-like cells.

j. Incubate the tube on ice for 20 min. Tap the tube gently every 10 min.
k. Add 1 mL of antibody buffer to dilute the antibody.
l. Centrifuge the cell suspension at 200 × *g* for 5 min.
m. Aspirate the supernatant and dislodge the pellet by rigorous tapping.
n. Resuspend the cells in 200 μL of antibody buffer.
o. Transfer the cell suspension through a cell strainer with 35 μm pore size to a 5 mL tube.
p. Keep the tube on ice until application to FACS.

17. Sort spermatogonium-like cells to establish multiclonal GSCLCs from a single rTestis (Figure 8).
    a. Sort the $VR^+CD9\text{-}APC^+$ cells (Figure 8A (lower left), P4 population) (step 16) by using a fluorescence-activated cell sorter (FACS) according to the manufacturer's instructions. We use FACS Aria III (BD Bioscience).
    b. Collect the sorted cells into a 15 mL tube containing 10 mL of wash buffer.
    c. Record the number of sorted cells as indicated in the "sort layout".

⚠ CRITICAL: To obtain a high-purity cell fraction, we select the "Purity" setting of the "Sort Precision Mode". To maintain the viability of sorted cells, we cool the collection tube holder by using the water bath system of the BD Temperature Control Option.

    d. Centrifuge the tube at 220 × *g* for 5 min.
    e. Aspirate the supernatant and dislodge the pellet by rigorous tapping.
    f. Resuspend the pellet in 100 μL of the GSC culture medium.
    g. Transfer 100 μL of the $VR^+CD9\text{-}APC^+$ spermatogonium-like cell suspension into the well of a 96-well plate (flat-bottom) with MEFs (step 14) (200 μL/well in total).
    h. Incubate the plate at 37°C in 5% CO_2, 95% air.

Note: Typically, 200–1000 cells/rTestis can be sorted.

18. Sort spermatogonium-like cells to establish monoclonal GSCLCs (Figure 8).
    a. Prepare 96-well plates (flat-bottom) with MEFs (step 14).
    b. Sort the $VR^+CD9\text{-}APC^+$ cells (Figure 8A (lower left), P4 population) (step 16) onto a 96-well plate by using a fluorescence-activated cell sorter (FACS) equipped with an automated cell deposition unit (ACDU) having a single cell plating setting according to the manufacturer's instructions. We use FACS Aria III (BD Biosciences).

⚠ CRITICAL: To obtain a high-purity cell fraction, we select the "Single cell" setting of the "Sort Precision Mode". To maintain the viability of sorted cells, we cool the collection tube holder by using the water bath system of the BD Temperature Control Option.

    c. Aspirate the serum medium and add GSC culture medium into the wells (200 μL/well in total).
    d. Centrifuge the 96-well plate at 100 × *g* for 3 min using a TOMY-CAX571 centrifuge to adhere the cells to the bottom of the wells.
    e. Incubate the plate at 37°C in 5% CO_2, 95% air.
19. Establish GSCLC lines (Figure 9).
    a. Prepare the GSC culture medium.
    b. Change half the volume of the GSC culture medium every 2 days.
    c. When one or a number of grape-shaped colonies are observed under an inverted microscope, passage all cells in one well of a 96-well plate to one well of a 48-well plate with MEF (see step 14).
    d. Similarly, passage progressively from a 48-well to a 24-well to a 12-well and to a 6-well plate.

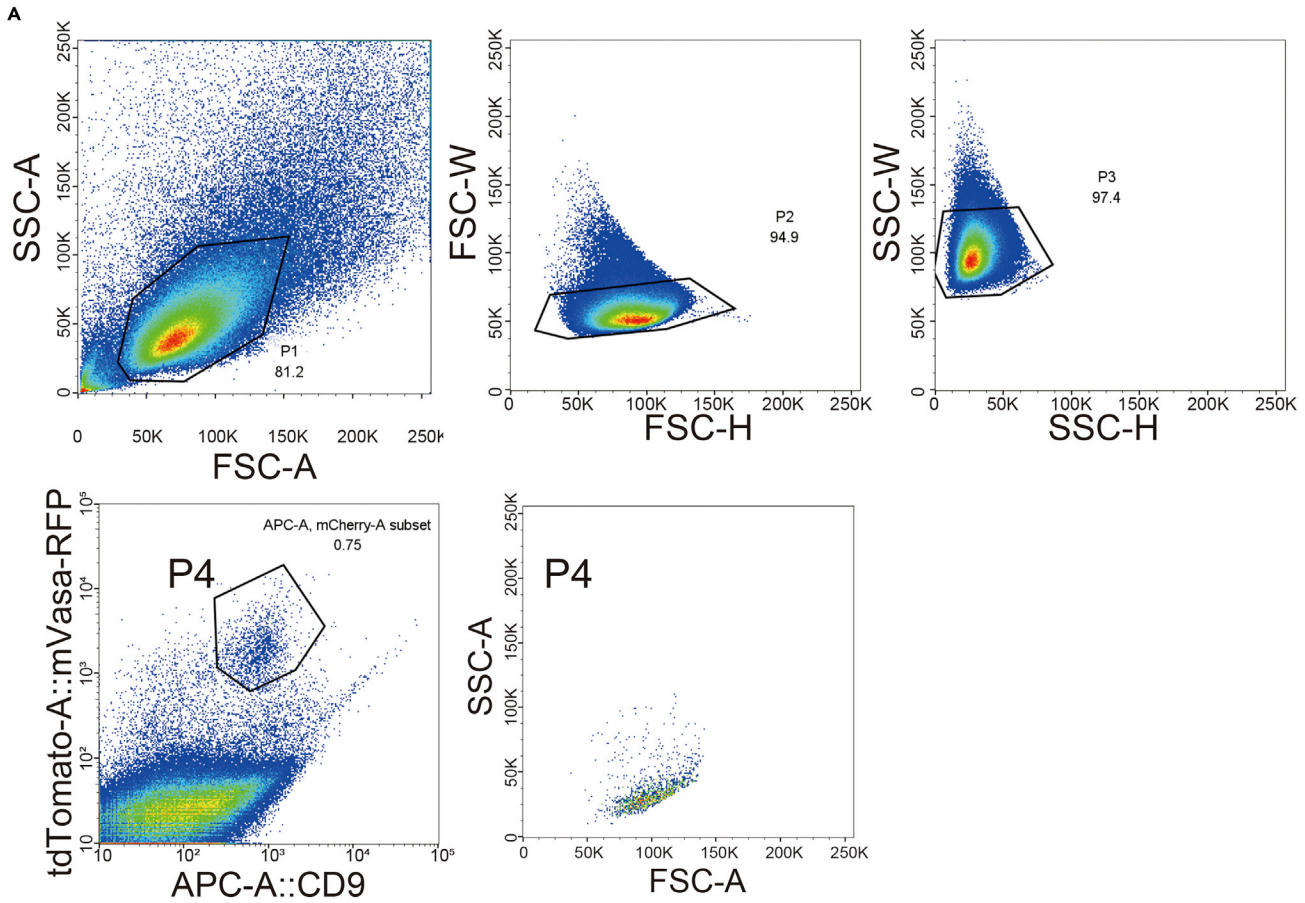

Figure 8. Purification of spermatogonium-like cells

(A) A flow cytometric gating strategy for sorting $CD9^+VR^+$ cells (spermatogonium-like cells) from an m14 rTestis. The P1 gate indicates depletion of dead cells (x-axis: FSC-A; y-axis: SSC-A) (upper left). The P2 gate is for single cell selection (x-axis: FSC-H; y-axis: FSC-W) (upper middle). The P3 gate is for single cell selection (x-axis: SSC-H; y-axis: SSC-W) (upper right). An example of $CD9^+VR^+$ cells sorting is shown (x-axis: VD9-APC; y-axis: VR) (lower left). The P4 gate for the $CD9^+VR^+$ cells is indicated. P4 population ($CD9^+VR^+$) cells are reflected in FSC-A; SSC-A dimension. Note that the $CD9^+VR^+$ cell population is located to the lower right of the P1 gate.

Note: From ~1–10^3 spermatogonium-like cells, GSCLC colonies with a typical grape-like colony morphology (Figure 9) are formed in ~2–3 weeks of culture. When grown up to a 6-well plate scale, the passage number is four and the number of cells reaches a 10^6-cell order. We consider that GSCLC lines are established when the cells grow stably and can be passaged more than five times. Prepare several freeze stocks at an early passage (step 21).

20. Passage GSCLCs (as an example, we will describe a passage from a 96-well to a 48-well plate).
    a. Prepare a 48-well plate (flat-bottom) with MEFs (see step 14).

    Note: Typical numbers of MEFs/well are as follows: 2×10^4 cells/96-well, 5×10^4 cells/48-well, 1×10^5 cells/24-well, 2×10^5 cells/12-well, 5×10^5 cells/6-well plate. The number of cells required depends on the viability of MEFs. A key point is that each well should be covered with MEFs without gaps.

    b. Prepare a 1.5 mL tube containing 500 μL/tube of the prewarmed wash buffer.
    c. Aspirate the medium of the wells of the 96-well plate with GSCLCs.
    d. Add 100 μL/well of prewarmed TrypLE Express, and incubate the plate at 37°C in 5% CO_2, 95% air for 4 min.

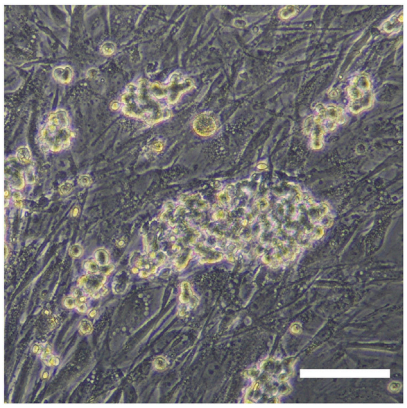

Figure 9. GSCLCs
A phase contrast image of mouse BVSCVR GSCLCs. Bar, 100 μm.

e. Dissociate the GSCLCs into single cells by pipetting them several times and transfer the cells into a 1.5 mL tube with a P200 pipette.
f. Add 100 μL/well of the prewarmed wash buffer to the wells, wash the wells thoroughly, and transfer the suspension to the 1.5 mL tube.
g. Centrifuge at 220 × *g* for 5 min to pellet the cells.
h. Aspirate the supernatant under a stereomicroscope to avoid accidental disposal of the cell pellet.
i. Resuspend the cells in 250 μL/tube of GSC culture medium.
j. Transfer the cell suspension to the well of a 48-well plate with MEFs.
k. Incubate the plate at 37°C in 5% CO_2, 95% air.

21. Prepare frozen stocks of GSCLCs.
    a. Passage GSCLCs up to a 6-well plate as described in step 20.

    Note: When ~2.0–3.0 × 10^5 GSCLCs are passaged into a well of a 6-well plate, they typically become confluent (~3.0–5.0 × 10^6 cells/well of a 6-well plate) after 7 days of culture. Accordingly, from one 6-well plate, ~2.0 × 10^7 GSCLCs can be harvested, and ~40 tubes (5 × 10^5 cells/tube) of GSCLC frozen stocks can be prepared.

    b. Aspirate the medium, add 1 mL of prewarmed TrypLE Express/well of a 6-well plate, and incubate the plate at 37°C in 5% CO_2, 95% air for 4 min.
    c. Dissociate the cells into single cells by pipetting them several times and transfer the cells into a 50 mL tube.
    d. Add 4 mL/well of the prewarmed wash buffer, wash the wells thoroughly, and transfer the suspension to the 50 mL tube.
    e. Count the number of the cells using a hemocytometer.
    f. Centrifuge the 50 mL tube at 220 × *g* for 3 min and aspirate the supernatant.
    g. Add CELLBANKER I plus so that the concentration of the cell suspension becomes ~1.0 × 10^6 cells/mL and transfer 500 μL to each cryotube (5.0 × 10^5 cells/tube).
    h. Store at −80°C.

EXPECTED OUTCOMES

Mouse ESCs can be induced into PGCLCs and then into spermatogonium/spermatogonial stem cell (SSC)-like cells in a manner that recapitulates spermatogonia/SSC development *in vivo*. The protocol can be applied to other mouse PSCs with a potential for PGCLC induction, including induced pluripotent stem cells (iPSCs) (Hayashi et al., 2011; Okita et al., 2007; Takahashi and Yamanaka, 2006) and stem cells with formative pluripotency (Kinoshita et al., 2020). The application to other mammals will require substantial protocol modifications/optimizations.

GSCLCs derived from spermatogonium/SSC-like cells show robust and stable expansion potential with the expression of key spermatogonia markers, such as *Nanos2*, *PLZF*, and *GFRa1*.

GSCLCs established under this protocol exhibit transcriptome and DNA methylome profiles highly similar to those of spermatogonia/SSCs or GSCs derived from spermatogonia/SSCs. Nonetheless, they do show differentially expressed genes (DEGs) and differentially methylated regions (DMRs) compared to spermatogonia/SSCs or GSCs, but such differences are, in most cases, to an extent that does not impact on the spermatogenic potential of GSCLCs (Ishikura et al., 2021).

GSCLCs differentiate into spermatozoa when transplanted into testes of germ cell-deficient mice, such as *W/W^v* mice, or testis transplants cultured *in vitro* (Ishikura et al., 2016, 2021; Sanjo et al., 2020; Sato et al., 2011). GSCLC-derived spermatozoa contribute to fertile offspring through in vitro fertilization or intracytoplasmic sperm injection.

LIMITATIONS

We use ESCs bearing transgene reporters for PGC (*Blimp1* and *Stella*) and male germ-cell differentiation (*Ddx4/mvh*) as a starting material for GSCLC derivation. Surface markers such as CD9 and MCAM for spermatogonium/SSC-like cell purification can be used. But other markers remain to be tested for robust and reproducible GSCLC derivation.

The PGCLC-to-spermatogonium/SSC-like cell differentiation in rTestes involves a substantial level of cell death, i.e., ~1,000 spermatogonium/SSC-like cells successfully differentiated from ~10,000 PGCLCs. The method for rTestis generation and culture requires further optimization.

GSCLCs derived under an optimal condition, while exhibiting a full capacity to differentiate into functional spermatozoa, still show some epigenetic differences, including those for DNA methylation profiles, from GSCs derived from spermatogonia/SSCs *in vivo*. Whether such epigenetic differences may have an impact on the physiology of the offspring remains to be determined.

TROUBLESHOOTING

Problem 1

ES cells do not grow under feeder-free culture conditions. (steps 36–39 in the "BEFORE YOU BEGIN" section).

Potential solution

Increase the number of cells initially applied to the well and thereby increase cell density.

Under feeder-free culture conditions, the cell density of ES cells is related to the viability of ES cells. When the cell density is low, ES cells tend to die.

Consider other cell lines.

The viability of ES cells may vary depending on the genetic background, the cell lines (even when the cell lines have the same genetic background), and the condition of the ES cells at the time the working stock is made.

Prepare a new 2iLIF with newly thawed N2B27 medium.

N2B27 medium should be used within 2 weeks after thawing and fresh medium should be used as much as possible.

Problem 2

ES cells do not adhere to the plate under feeder-free culture conditions. (steps 36–39 in the "BEFORE YOU BEGIN" section).

Potential solution

Increase the concentration of laminin.

Depending on the cell line, we will vary the laminin concentration in the range of 10–300 ng/mL to determine the concentration that is more conducive to adhesion and growth.

Single cell dissociation of ESCs.

Clumps of ES cells that are not dissociated to single cells have a smaller surface area for adherence to the laminin-coated plate, and thus these clumps can easily detach and engulf other ES cells, adversely affecting the culture and proliferation of ES cells.

Problem 3

PGCLCs cannot be induced with stable efficiency. (steps 1–4 in the "STEP-BY-STEP METHOD DETAILS" section).

Potential solution

Check the KSR concentration of the EpiLC induction medium.

During induction into EpiLCs, the concentration of KSR in the medium should be strictly checked to ensure that it does not exceed 1%.

KSR lot confirmation.

The induction efficiency of PGCLCs depends on the lot of KSR. If you get multiple KSR lots, it is recommended that PGCLC induction be performed and assessed separately for each lot.

Use a low passage number of ES cell lines.

As the number of ES cell passages increases, karyotypes become abnormal and aberrant cells become dominant, which causes abnormal PGCLCs induction.

Optimize the appropriate time point for EpiLC induction.

It is important to strictly determine the induction time of EpiLCs for each ESC line, because EpiLCs acquire the competence to differentiate into PGCLCs within a transient window during their differentiation, just as epiblast cells do *in vivo* (Hayashi et al., 2011). Typically, we start with 0.8–1 × 10^5 ESCs/well of a 12-well plate and determine the time required for the cells to grow to 1 × 10^6 cells/well of a 12-well plate (a sub-confluent state for the 12 wells). This usually takes 44–48 h.

Check the number of cells in the PGCLC aggregates.

Typically, the number of cells we use is 2000–5000 cells/aggregate.

Problem 4

Low efficiency of PGCLC expansion. (steps 5–7 in the "STEP-BY-STEP METHOD DETAILS" section).

Potential solution

The expansion rate of PGCLCs is critical for their differentiation into spermatogonium/SSC-like cells in rTestes, because PGCLCs undergo replication-coupled genome-wide DNA demethylation, including promoter demethylation, from genes essential for male germ-cell differentiation.

Check the induction time of PGCLCs.

The expansion potential of PGCLCs depends on the induction time of PGCLCs, and therefore, an appropriate PGCLC induction time should be determined for each ESC line. For example, as aforementioned, the BVSCVR ESC-derived PGCLCs show an appropriate expansion potential at ~90 h after PGCLC induction, at which time PGCLCs show a robust BV fluorescence, but have only just initiated SC fluorescence (Figure 5E). Fully BV^+ and SC^+ PGCLCs show a low expansion potential.

Check the concentration of m220-5 and d4PGCLC for PGCLC culture.

The quality of m220-5 feeders is also critical for an appropriate PGCLC expansion. Determine and confirm the appropriate preparation of the m220-5 feeders (this depends on the batch of m220-5 feeder stocks) with the optimal MMC concentration (~1–2 μg/mL). Also, make sure that the concentrations of m220-5 feeders (~2.5×10^5 cells to a 1 cm^2 area) and PGCLCs (~2.5×10^3 cells to a 1 cm^2 area) are appropriate for proper PGCLC expansion.

Check the quality of m220-5 stock.

Ensure that MMC treatment of m220-5 cells is adequate. Specifically, check that m220-5 cells do not proliferate in culture.

Problem 5

Dedifferentiation of PGCLCs during the expansion culture. (steps 5–7 in the "STEP-BY-STEP METHOD DETAILS" section).

Potential solution

Sort d4PGCLCs into the appropriate gate.

More stringent gating of BV-positive cells to avoid contamination with BV-negative cells. (step 6 in the "STEP-BY-STEP METHOD DETAILS" section).

Select another genetic background of ESCs.

Some genetic backgrounds are prone to de-differentiation and proliferation of ES cell-like cells.

Insufficient EpiLCs induction.

Typically, we culture EpiLCs from 44 h to 48 h; less than 44 h is not recommended. (steps 2–4 in the "STEP-BY-STEP METHOD DETAILS" section).

Problem 6

The culture fluid exudes from under the membrane during reconstituted testis culture. (step 13 in the "STEP-BY-STEP METHOD DETAILS" section).

Potential solution

When changing the culture medium, do not replace more than half the medium at one time, and carefully monitor the condition of the rTestes.

A volume of medium equivalent to 340–350 μL of the rTestis medium is replaced. The volume of the medium should not be higher than this standard. If any medium seeps over the membrane, carefully remove it using a P200 pipette.

Problem 7

In functional analysis of GSCLCs, no sperm differentiation is observed.

Potential solution

Check the ESCs/iPSCs quality.

Please make sure that the karyotype of the mouse ESC/iPSC is 40XY.

Check the expanded PGCLCs quality.

Please confirm the PGCLC expansion (check the fold change, typically an over 8-fold increase in proliferation) (steps 5–7).

Check the rTestes culture quality.

The rTestis culture must be done properly (after 14 days of culture, sorted cells should be in the range of 200–1000 cells/rTestis). (steps 8–13 in the "STEP-BY-STEP METHOD DETAILS" section).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mitinori Saitou (saitou@anat2.med.kyoto-u.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The data generated or analyzed during this study are included in this article.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.I., H.O., and M.S.; methodology, Y.I., H.O., and M.N.; investigation, Y.I., H.O., and M.N.; writing, Y.I., H.O., M.N., and M.S.

DECLARATION OF INTERESTS

Y.I., H.O., and M.S. are inventors on patent applications relating to the induction of germ cells from pluripotent stem cells filed by Kyoto University.

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