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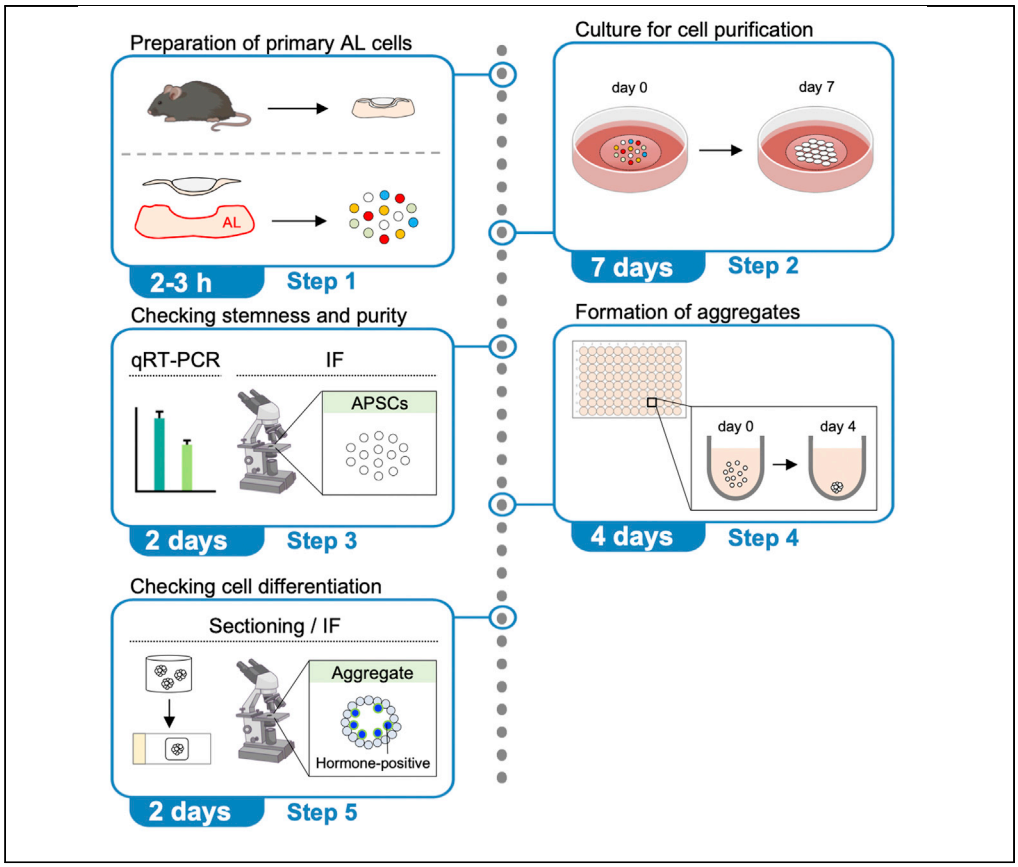
Protocol for isolating adult pituitary stem/progenitor cells in mice



Hormone-producing cells in the anterior lobe (AL) of the pituitary gland are important for growth and reproduction, but it has been challenging to isolate their source: the pituitary stem/progenitor cells. Here, we present a protocol for isolating adult pituitary stem/progenitor cells (APSCs) in mice. We describe dissociation and culture of AL cells, followed by the assessments of stemness marker expression and the differentiation capacity. This protocol enables separation of APSCs based on their cell adhesion properties with nearly 100% purity.

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

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Highlights

We describe primary adult mouse pituitary stem/progenitor cell (APSC) isolation

This protocol is capable of separating APSCs at almost 100% purity levels

Isolated APSCs are functional for down-stream applications

This technique does not require difficult-to-use reagents or any special equipment

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Protocol for isolating adult pituitary stem/progenitor cells in mice

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SUMMARY

Hormone-producing cells in the anterior lobe (AL) of the pituitary gland are important for growth and reproduction, but it has been challenging to isolate their source: the pituitary stem/progenitor cells. Here, we present a protocol for isolating adult pituitary stem/progenitor cells (APSCs) in mice. We describe dissociation and culture of AL cells, followed by the assessments of stemness marker expression and the differentiation capacity. This protocol enables separation of APSCs based on their cell adhesion properties with nearly 100% purity. For complete details on the use and execution of this protocol, please refer to Shintani and Higuchi (2021).

BEFORE YOU BEGIN

This protocol describes the procedure for isolating pituitary stem/progenitor cells from healthy, freshly extracted pituitary gland, specifically the anterior lobe (AL) of the pituitary gland, in adult mice.

All animal experiments were approved by the Institutional Animal Care and Use Committee of Tottori University and were conducted in accordance with the NIH Guidelines for Animal Care and Use of Laboratory Animals.

We typically isolated the pituitary stem/progenitor cells from approximately three mice per experiment. However, it is also possible to perform this protocol on more than two but less than five mice.

Minimizing the operation time, removing the intermediate lobe/posterior lobe (IL/PL) as much as possible, using an appropriate medium for cell adhesion (AM), seeding primary cells at high density in glass-bottom dishes, and removing as much AM as possible when replacing the medium with stem cell growth medium (GM) are pivotal for the success of isolating mouse adult pituitary stem/progenitor cells.

Therefore, all solutions and equipment listed below and in the accompanying key resources table should be prepared and ready for use before dissection.

All materials required are listed in the attached key resources table and in the materials and equipment section.

Instrument sterilization

Sterilize the dissecting instruments with 75% (v/v) ethanol before use.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Isoflurane	FUJIFILM Wako	Cat#099-06571
EtOH	Japan Alcohol Corporation	N/A
HEPES	Dojindo	Cat#GB10
NaCl	FUJIFILM Wako	Cat#191-01665
EDTA	Dojindo	Cat#N001
NaOH	Nacalai Tesque	Cat#37420-15
Triton X-100	Nacalai Tesque	Cat#35501-15
Trehalose	FUJIFILM Wako	Cat#206-18455
Collagenase, *Clostridium histolyticum*	Sigma-Aldrich	Cat#C9263
Trypsin from Porcine Pancreas	Nacalai Tesque	Cat#35547-64
DNase I	Promega	Cat#M6101
DMEM	Nacalai Tesque	Cat#08458-45
DMEM/Ham's F-12	Nacalai Tesque	Cat#08460-95
B27-supplement (50 ×), minus vitamin A	Thermo Fisher Scientific	Cat#12587010
Recombinant Mouse bFGF Protein	R&D Systems	Cat#3139-FB-025
Recombinant Mouse EGF Protein, Carrier-free	R&D Systems	Cat#2028-EG-200
Fetal bovine serum (FBS)	Sigma	Cat#176012-500ML
Bovine serum albumin (BSA), Fatty acid free	FUJIFILM Wako	Cat#017-22231
Horse serum (HS), New Zealand origin	Thermo Fisher Scientific	Cat#16050122
VECTASHIELD Mounting Medium with DAPI	Vector	Cat#H-1200
Normal donkey serum	Jackson ImmunoResearch	Cat#017-000-121
Paraformaldehyde (PFA)	FUJIFILM Wako	Cat#162-16065
RNase Inhibitor, Recombinant	Toyobo	Cat#SIN-201
Tissue-Tek® O.C.T. Compound	Sakura Finetek Japan	Cat#4583
PrimeScript II Reverse Transcriptase	Takara	Cat#2680A
dNTP Mixture	Takara	Cat#4030
Oligo dT primer (×15T)	Fasmac	N/A
SYBR® Green Realtime PCR Master Mix -Plus-	Toyobo	Cat#QPK-212
Oligonucleotides		
Primer: *Prrx1*-F: 5′-TTACCCGGATGCTTTTGTTC-3′	Shintani and Higuchi (2021)	N/A
Primer: *Prrx1*-R: 5′-ACTTGGCTCTTCGGTTCTGA-3′	Shintani and Higuchi (2021)	N/A
Primer: *Sox2*-F: 5′-CCGTTTTCGTGGTCTTGTTT-3′	Shintani and Higuchi (2021)	N/A
Primer: *Sox2*-R: 5′-TCAACCTGCATGGACATTTT-3′	Shintani and Higuchi (2021)	N/A
Primer: *Pitx1*-F: 5′-CTCTGCTCCACTCTGGGTC-3′	Shintani and Higuchi (2021)	N/A
Primer: *Pitx1*-R: 5′-GTCCGACATACACAGGGACG-3′	Shintani and Higuchi (2021)	N/A
Primer: *Cdh1*-F: 5′-CCTGCCAATCCTGATGAAAT-3′	Shintani and Higuchi (2021)	N/A
Primer: *Cdh1*-R: 5′-CGAACACCAACAGAGAGTCG-3′	Shintani and Higuchi (2021)	N/A
Primer: *Pomc*-F: 5′-TAGATGTGTGGAGCTGGTGC-3′	Shintani and Higuchi (2021)	N/A
Primer: *Pomc*-R: 5′-CGTACTTCCGGGGGTTTTCA-3′	Shintani and Higuchi (2021)	N/A
Primer: *Lhb*-F: 5′-CCGGCTACTGTCCTAGCAT-3′	Shintani and Higuchi (2021)	N/A
Primer: *Lhb*-R: 5′-GGAAAGGAGACTATGGGGTCT-3′	Shintani and Higuchi (2021)	N/A
Primer: *Tshb*-F: 5′-CCTGACCATCAACACCACCA-3′	Shintani and Higuchi (2021)	N/A
Primer: *Tshb*-R: 5′-TATGGCGACAGGGAAGGAGA-3′	Shintani and Higuchi (2021)	N/A
Primer: *Gh*-F: 5′-ACCTCGGACCGTGTCTATGA-3′	Shintani and Higuchi (2021)	N/A
Primer: *Gh*-R: 5′-CATGTTGGCGTCAAACTTGT-3′	Shintani and Higuchi (2021)	N/A
Primer: *Prl*-F: 5′-GGTCATCAATGACTGCCCCA-3′	Shintani and Higuchi (2021)	N/A
Primer: *Prl*-R: 5′-CTCGAGGACTGCACCAAACT-3′	Shintani and Higuchi (2021)	N/A
Primer: *Tbp*-F: 5′- GATCAAACCCAGAATTGTTCTCC-3′	Shintani and Higuchi (2021)	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Primer: *Tbp*-R: 5′-ATGTGGTCTTCCTGAATCCC-3′	Shintani and Higuchi (2021)	N/A
Experimental models: Organisms/strains		
Mouse: C57BL/6J, 8–12 weeks old, male or female	CLEA Japan	N/A
Antibodies		
Anti-human PRRX1 (dilution 1: 100), Rabbit	Novus Biologicals	Cat#NBP2-13816
Anti-human SOX2 (dilution 1: 400), Goat	Neuromics	Cat#GT15098
Anti-human ACTH (dilution 1: 5,000), Guinea pig	kindly provided by Dr. Shigeyasu Tanaka (Shizuoka University)	N/A
Anti-rat LHβ (dilution 1: 5,000), Guinea pig	NIDDK	N/A
Anti-rat TSHβ (dilution 1: 20,000), Guinea pig	NIDDK	N/A
Anti-human GH (dilution 1: 2,000), Guinea pig	kindly provided by Dr. Shigeyasu Tanaka (Shizuoka University)	N/A
Anti-rat PRL (dilution 1: 10,000), Guinea pig	NIDDK	N/A
Donkey anti-rabbit IgG, Cy3-conjugated (dilution 1: 500)	Jackson ImmunoResearch	Cat#711-166-152
Donkey anti-guinea pig IgG, AlexaFluor488-conjugated (dilution 1: 500)	Jackson ImmunoResearch	Cat#706-546-148
Donkey anti-goat IgG, AlexaFluor647-conjugated (dilution 1: 500)	Jackson ImmunoResearch	Cat#705-606-147
Critical commercial assays		
NucleoSpin RNA Plus XS	MACHEREY-NAGEL	Cat#U0990B
iPGell	Geno Staff	Cat#PG20-1
Software and algorithms		
CFX Maestro Software	Bio-Rad	N/A
cellSens dimension system	Olympus	N/A
Other		
CFX Connect Real-Time PCR Detection System	Bio-Rad	N/A
IXplore Pro	Olympus	N/A
ECLIPSE Ts2-FL	Nikon	N/A
Tabletop Centrifuge	KUBOTA	Cat#2410
Tabletop Micro Refrigerated Centrifuge	KUBOTA	Cat#3520
NanoDrop	Thermo Fisher Scientific	N/A
Dissecting instruments (scissors and forceps)	N/A	N/A
Millex-GP Syringe Filter Unit, 0.22 μm	Merck Millipore	Cat#SLGPR33RS
U-bottom 96-well plates	Sumitomo Bakelite	Cat#M9096U
CELLview Cell Culture Dish with Glass Bottom	Greiner Bio-One	Cat#627860
35-mm plastic dish	IWAKI	Cat#1000-035
CO_2 incubator	Astec	Cat#SMA-80DS
Hard-Shell® 96-Well PCR Plates, low profile, thin wall	Bio-Rad	Cat#HSP9655
Low-Profile PCR Tubes 8-tube strip	Bio-Rad	Cat#TLS0851
0.2 mL Flat PCR Tube 8-Cap Strips	Bio-Rad	Cat#TSC0803
MAS-coated slide glass	Matsunami Glass	Cat#SMAS-01

MATERIALS AND EQUIPMENT

Reagent preparation

- 75% (v/v) EtOH. Dilute 75 mL of 99.8% EtOH in 25 mL of sterile distilled water. Store at 20°C–25°C for up to 1 month.
- 1 M HEPES buffer (pH 7.5). Add 119.15 g of HEPES to 400 mL of distilled water. Dissolve the reagent at 20°C–25°C, and then adjust the pH to 7.5 using 4 N NaOH. Use distilled water to bring the final volume to 500 mL and store at 20°C–25°C for up to 6 months after autoclaving.
- 5 M NaCl. Add 146.1 g of NaCl to 400 mL of distilled water. Dissolve the reagent at 20°C–25°C. Use distilled water to bring the final volume to 500 mL and store at 20°C–25°C for up to 6 months after autoclaving.

- 0.5 M EDTA. Add 93.06 g of EDTA 2Na•2H_2O and 10 g of NaOH to 400 mL distilled water. Dissolve reagents at 20°C–25°C, and then adjust the pH to 8.0 using 4 N NaOH. Use distilled water to bring the final volume to 500 mL and then sterilize the solution by passing through a 0.22 μm filter. Stored at 20°C–25°C for up to 6 months.
- 50 mM EDTA. Dilute 10 mL of 0.5 M EDTA in 90 mL of sterile distilled water. Store at 20°C–25°C for up to 1 month.
- 20% (v/v) Triton X-100. Dilute 10 mL of 100% Triton X-100 in 40 mL of sterile distilled water. Store at 20°C–25°C for up to 6 months.
- 30% (w/v) bovine serum albumin (BSA). Aliquot 1 mL of the thawed 30% BSA and store at −30°C for up to 6 months.
- B27-supplement. Aliquot 1 mL of the thawed B27-supplement (50×) and store at −30°C for up to 2 months.

2% (w/v) collagenase solution

Reagent	Final concentration	Amount
Collagenase	2%	50 mg
HEPES buffer (1 M)	20 mM	50 μL
ddH_2O	n/a	2.450 mL
Total	**n/a**	**2.5 mL**

Sterilize the solution by passing through a 0.22 μm filter. Aliquot 833 μL and store at −30°C for up to 2 months.

2.5% (w/v) trypsin solution

Reagent	Final concentration	Amount
Trypsin	2.5%	250 mg
HEPES buffer (1 M)	20 mM	0.2 mL
ddH_2O	n/a	9.8 mL
Total	**n/a**	**10 mL**

Sterilize the solution by passing through a 0.22 μm filter. Aliquot 220 μL and stored at −30°C for up to 2 months.

bFGF solution

Reagent	Final concentration	Amount
Recombinant mouse bFGF	100 μg/mL	25 μg
BSA (30%)	0.1%	0.83 μL
HEPES buffer (1 M)	20 mM	5 μL
ddH_2O	n/a	244.17 μL
Total	**n/a**	**250 μL**

Aliquot 25 μL of each reagent and store at −30°C for up to 3 months.

EGF solution

Reagent	Final concentration	Amount
Recombinant mouse EGF	200 μg/mL	200 μg
BSA (30%)	0.1%	3.3 μL
HEPES buffer (1 M)	20 mM	20 μL
ddH_2O	n/a	976.7 μL
Total	**n/a**	**1 mL**

Aliquot 65 μL of each reagent and store at −30°C for up to 3 months.

4% (w/v) paraformaldehyde (PFA) solution		
Reagent	Final concentration	Amount
Paraformaldehyde	4%	4 g
NaOH (4 N)	5 mM	125 µL
HEPES buffer (1 M)	20 mM	2 mL
ddH_2O	n/a	up to 100 mL
Total	**n/a**	**100 mL**
Because of the high fixation capacity of fresh solutions, PFA solution is prepared on demand.		

Note: Add PFA to approximately 80 mL sterile distilled water. Dissolve the reagent at 60°C after adding 4 N NaOH, add 1 M HEPES buffer, and filter the solution through a filter paper. Use distilled water to bring the final volume to 100 mL and store at 4°C until use.

30% (w/v) trehalose solution		
Reagent	Final concentration	Amount
Trehalose	30%	30 g
HEPES buffer (1 M)	20 mM	2 mL
ddH_2O	n/a	up to 100 mL
Total	**n/a**	**100 mL**
Store at 4°C for up to 1 month.		

Note: Add trehalose to approximately 50 mL sterile distilled water and dissolve the reagent at 20°C–25°C. Add 1 M HEPES buffer and use sterile distilled water to bring the final volume to 100 mL.

HEPES-buffered saline solution (HBSS)		
Reagent	Final concentration	Amount
HEPES buffer (1 M)	20 mM	20 mL
NaCl (5 M)	100 mM	20 mL
ddH_2O	n/a	960 mL
Total	**n/a**	**1,000 mL**
Store HBSS at 20°C–25°C for up to 1 month after autoclaving.		

HEPES-T (for blocking, permeabilization, and dilution of antibodies)		
Reagent	Final concentration	Amount
HEPES buffer (1 M)	20 mM	1 mL
NaCl (5 M)	100 mM	1 mL
Triton X-100 (20%)	0.4%	1 mL
ddH_2O	n/a	47 mL
Total	**n/a**	**50 mL**
Store HEPES-T at 4°C for up to 6 months.		

Digestion buffer A		
Reagent	Final concentration	Amount
HBSS	1 ×	4.45 mL
Trypsin solution (2.5%)	0.25%	500 µL
EDTA (50 mM)	0.5 mM	50 µL
Total	**n/a**	**5 mL**
Prepare on demand. Bring to 20°C–25°C before use.		

Digestion buffer B		
Reagent	Final concentration	Amount
HBSS	1 ×	4.9 mL
Trypsin solution (2.5%)	0.025%	50 μL
EDTA (50 mM)	0.5 mM	50 μL
Total	**n/a**	**5 mL**
Prepare on demand. Bring to 20°C–25°C before use.		

Culture media preparation

Cell adhesion medium (AM)		
Reagent	Final concentration	Amount
DMEM	1 ×	440 mL
FBS	10%	50 mL
HEPES buffer (1 M)	20 mM	10 mL
Total	**n/a**	**500 mL**
Sterilize AM by passing through a 0.22 μm filter and store at 4°C for up to 1 month. Bring to 20°C–25°C before use.		

Stem cell growth medium (GM)		
Reagent	Final concentration	Amount
DMEM/F-12	1 ×	9.897 mL
B27-supplement (50×), minus vitamin A	0.5 ×	100 μL
Recombinant mouse bFGF (100 μg/mL)	20 ng/mL	2 μL
Recombinant mouse EGF (200 μg/mL)	20 ng/mL	1 μL
Total	**n/a**	**10 mL**
Prepare on demand. Bring to 20°C–25°C before use.		

Differentiation medium (DM)		
Reagent	Final concentration	Amount
DMEM/F-12	1 ×	437.5 mL
HS	10%	50 mL
FBS	2.5%	12.5 mL
Total	**n/a**	**500 mL**
Sterilize DM by passing through a 0.22 μm filter and store at 4°C for up to 1 month. Bring to 20°C–25°C before use.		

STEP-BY-STEP METHOD DETAILS

Dissociation of AL from the pituitary gland in adult mice

Timing: approx. within 10 min/mouse

This section describes how to crack the skull and extract the pituitary gland. An overview of the procedure for pituitary gland extraction is shown in Figure 1.

1. Euthanize the mice by isoflurane overexposure.
2. Spray with 75% (v/v) ethanol to generate a sterile environment, followed by decapitation.
3. Using sterile surgical tools, peel the head skin between the ears.
4. Insert scissors toward the ears (along the dotted line) to open the skull using the principle of leverage.

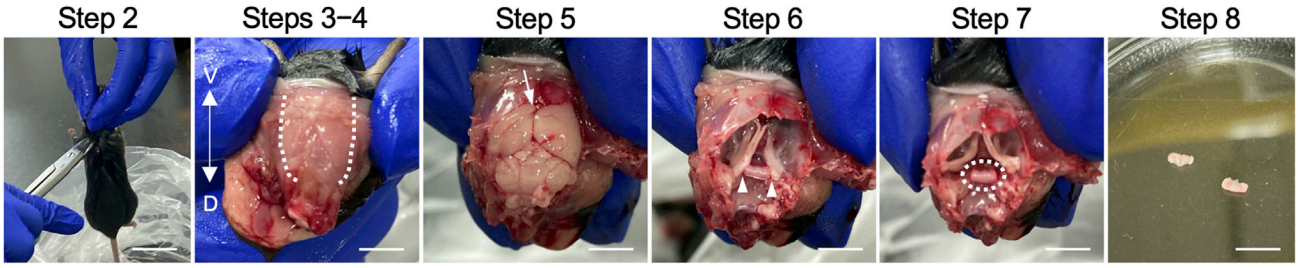

Figure 1. Dissection of pituitary gland from mouse

Step 2: Beheading. Steps 3–4: Removal of skull. Dotted lines indicate the positions to be cut. V: ventral side. D: dorsal side. Step 5: Removal of brain. The tip of the arrow indicates the position to insert the medicine spoon. Step 6: Detachment of the *diaphragma sellae*. The arrows indicate where to pinch with tweezers. Step 7: The circle indicates the isolated pituitary tissue. Step 8: Excised pituitary glands. Scale bars: 20 mm (step 2), 5 mm (steps 3–8).

5. Gently insert the medicine spoon between the brain and skull (the tip of the arrow) and scrape out the brain.
6. Remove the *diaphragma sellae* with sharp tweezers without damaging the pituitary gland.

   Note: At both ends (the tip of arrowheads) of the pituitary gland, there is a thin membrane that can be pinched with tweezers, so pinch them and flip them in two places toward the ventral side back to remove the *diaphragma sellae*.

7. Trim the surrounding tissue to make it easier to access the pituitary gland (in the circle).
8. Gently grasp the pituitary gland with blunt tweezers and transfer to a 35-mm plastic dish filled with 2 mL of HBSS.
9. Discard the IL and PL from the pituitary gland under a stereomicroscope (Figure 2).

   Note: The IL is transparent to light, so it can be used as a guide to stick the tip of the sharp tweezers into the remnant cavity at the border of the AL.

10. Transfer the AL to a new 35-mm plastic dish filled with 2 mL of fresh HBSS and carefully isolate with sharp tweezers (split into two) to increase the surface area for the enzymatic reaction.
11. Proceed to the next step after collecting three individuals.

Preparation of a single-cell suspension of primary AL cells

Timing: approx. within 60 min

This section describes steps for the dispersion of primary cells from the AL.

12. Collect the AL fragments in a new 35-mm plastic dish filled with 1.8 mL of AM.
13. Add 0.2 mL of 2% collagenase solution and mix by pipetting.
14. Transfer the AL fragments (with AM) to a 15-mL conical tube using a micropipette (P1000) and incubate in a water bath at 37°C for 15 min. Mix by tapping the conical tube, once every 5 min.
15. Without removing the supernatant, add 5 μL of DNase I solution. Mix by tapping the conical tube 5 times, followed by incubation at 37°C for 5 min.
16. Centrifuge at 100 × *g* for 5 min at 20°C–25°C and remove the supernatant using a pipette.
17. Add 2 mL of digestion buffer A, followed by incubation at 37°C for 5 min.
18. Add 2 mL of AM to stop the trypsin reaction.
19. Centrifuge at 100 × *g* for 5 min at 20°C–25°C and remove the supernatant using a pipette.
20. Add 2 mL of fresh AM and then dissociate the AL into single cells. Specifically, aspirate and expel the AL with a micropipette (dial is set to 1,000 μL) multiple times (approximately 10–20 times) until fragments are no longer visible.

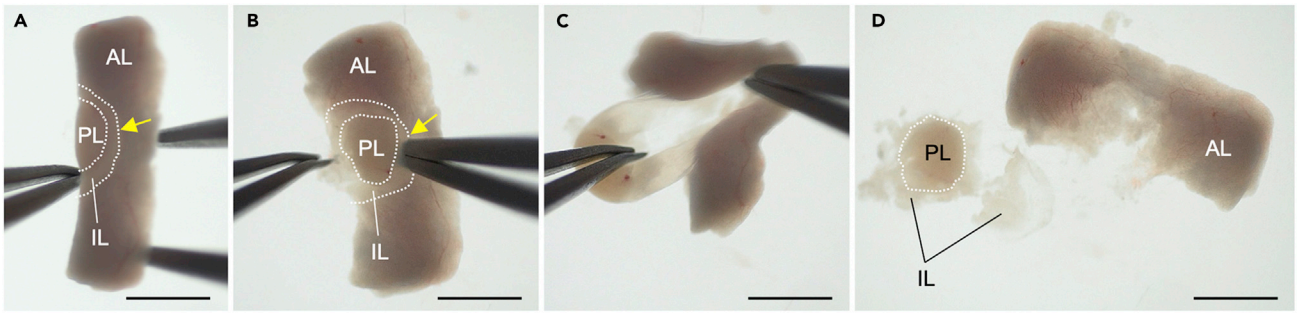

Figure 2. Separation of anterior lobe (AL) and intermediate lobe (IL)/posterior lobe (PL)
(A) Dorsal view of the pituitary gland. Dotted lines indicate the boundary between the AL and the IL or the IL and PL. The tip of the arrow indicates the Rathke's cleft.
(B) Poke the tip of the tweezers into the border area between the AL and the IL.
(C) Using the pierced tweezers as a support, peel off the IL/PL with the opposite tweezers.
(D) Image of the AL and the IL/PL after separation. Scale bars: 1 mm.

21. Count the number of primary AL cells.

Two-dimensional (2D) culture of primary AL cells

Timing: approx. 7 days

This section describes steps for initial growth and expansion of primary AL cells.

22. Seed at 75,000 cells/500 μL on the glass surface (4.52 cm^2) of a glass-bottom dish (Figure 3). Transfer to a CO_2 incubator (5% CO_2/95% air) and preculture at 37°C for 30–60 min.

⚠ CRITICAL: If the volume exceeds 500 μL, the drop will overflow and break down.

⚠ CRITICAL: When a plastic dish is used instead of a glass-bottom dish for cell culture, the cells will grow but the cell density will remain low even after 7 days (Figure 4).

23. Add 1.5 mL of fresh AM to a glass-bottom dish with a drop, and incubate until the next day.

⚠ CRITICAL: If GM is used from the beginning, the cells will hardly stick to it and will not proliferate.

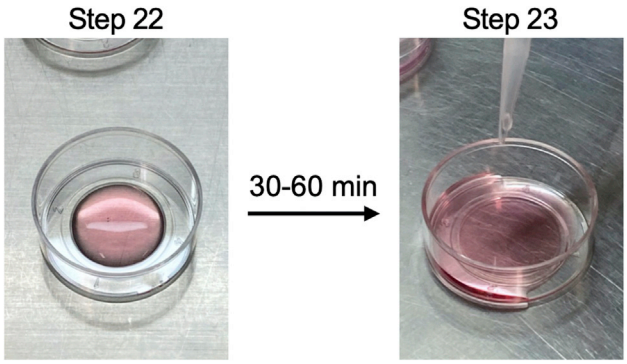

Figure 3. Points to note while seeding the primary cells
Step 22: Make a droplet so that the culture fluid does not overflow from the glass surface of the glass-bottom dish.
Step 23: Drip the culture medium from around the droplet.

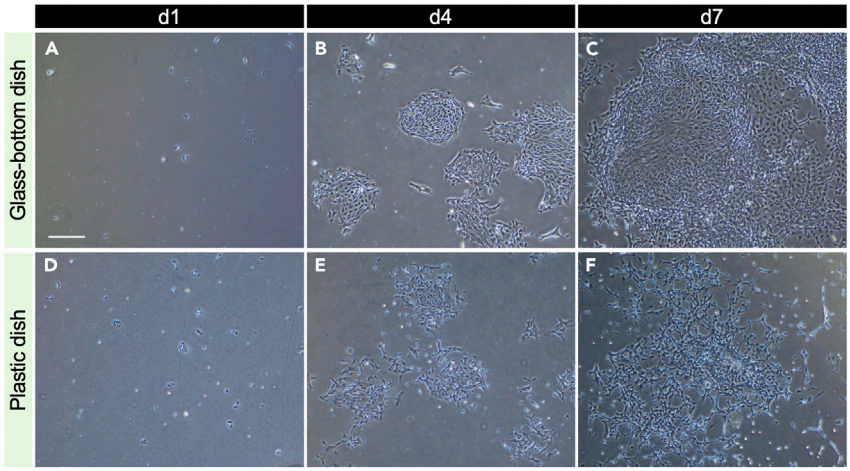

Figure 4. Growth of primary anterior lobe (AL) cells isolated from mouse pituitary gland

Representative phase contrast images are shown in the cell culture on a glass-bottom dish (A–C) or a plastic dish (D–F).

(A and D) Primary AL cells immediately after medium change (day 1 of culture) (d1).

(B and E) Primary AL cells on day 4 of culture (d4).

(C and F) Primary AL cells on day 7 of culture (d7). Scale bar: 300 μm.

⚠ CRITICAL: If you need to check the purity of the adherent cells, perform steps 38 through 47 using cells from the day after seeding. If approximately 80% of the cells are PRRX1-positive at this point, cell selection is proceeding appropriately (Figure 5).

24. Replace with GM and incubate for another 6 days. Change the entire culture medium every three days.
    a. Remove the culture medium using a micropipette (P1000) for changing the medium, and rinse twice with 1 mL of HBSS.
    b. Add 2 mL of GM and incubate in a CO_2 incubator (5% CO_2/95% air) at 37°C.
25. Proceed to the next step after a total of 7 days of cell culture. Photograph cells using ECLIPSE Ts2-FL (Figure 4).

Gene expression profiles of primary-cultured AL cells by quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR)

Timing: approx. 5 h

This section describes steps for the analysis of gene expression levels in primary-cultured AL cells.

26. Proceed up to step 25.
27. Remove the culture medium using a micropipette (dial is set to 1,000 μL) and then rinse twice with 1 mL of HBSS.
28. Lyse cells by adding 100 μL of LB1 (One of the components of the NucleoSpin RNA Plus XS kit) onto the glass surface of a glass-bottom dish for 5 min at 20°C–25°C.
29. Extract the total RNA using the NucleoSpin RNA Plus XS kit according to the manufacturer's instructions and measure its concentration using an absorption photometer such as NanoDrop.
30. Synthesize complementary DNA (cDNA) from 250–500 ng of total RNA.
    a. Prepare the following mixture in a microtube.

Reagent	Amount
dNTP Mixture (2 mM)	4 μL
Oligo dT primer (50 μM)	1 μL
Template total RNA	250 ng ≦ total RNA ≦ 500 ng
ddH_2O	up to 10 μL

b. Heat at 65°C for 5 min and cool immediately on ice.
c. Prepare the reaction mixture by combining the following to a total volume of 20 μL.

Reagent	Amount
Template RNA/Primer mixture	10 μL
PrimeScript II RTase	1 μL
5× PrimeScript II buffer	4 μL
RNase Inhibitor, Recombinant	0.5 μL
ddH_2O	4.5 μL

d. Perform the reaction at 42°C for 60 min.
e. Heat at 70°C for 15 min and cool on ice.

31. Dilute with sterile water to a concentration of cDNA derived from 10 ng/μL total RNA equivalent. Store at −30°C until use.
32. Prepare the following premix for qRT-PCR.

Reagent	Amount
SYBR® Green Realtime PCR Master Mix	5 μL
Plus solution	1 μL
Forward primer (10 μM)	0.5 μL
Reverse primer (10 μM)	0.5 μL
ddH_2O	2.5 μL
Total	**9.5 μL**

33. Add 9.5 μL of the premix followed by 0.5 μL of diluted cDNA to 8-stripe tubes or a 96-well plate, mix, and centrifuge briefly.
34. Perform qRT-PCR using a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol of the SYBR Green Real-time PCR Master Mix Plus (Toyobo Co., Ltd., Osaka, Japan).
35. Calculate the relative gene expression by comparing cycle times for each targeted PCR.
36. Normalize target gene expression levels using TATA-box-binding protein (*Tbp*) as an internal standard gene (ΔCt).

Checking the stemness marker of primary-cultured AL cells by immunofluorescence analysis

Timing: approx. 2 days

This section describes steps for immunofluorescence analysis of primary-cultured AL cells.

37. Proceed up to step 25.
38. Remove the culture medium using a micropipette, and then rinse twice with 1 mL of HBSS.

Note: All further operations should be performed in a humidified chamber to prevent drying.

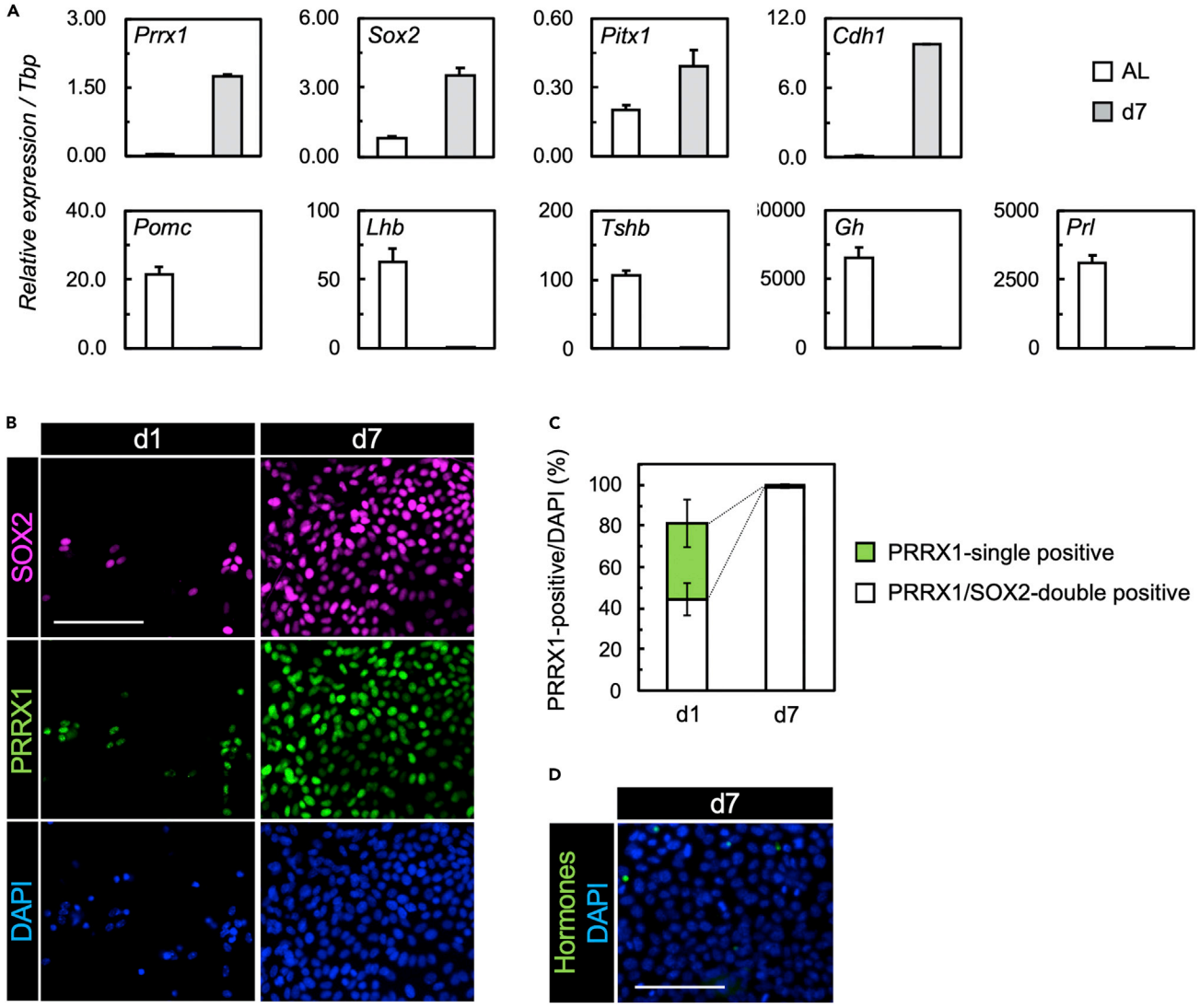

Figure 5. Quality check for primary-cultured anterior lobe (AL) cells isolated from the mouse pituitary gland

(A) Primary-cultured AL cells were subjected to qRT-PCR analysis of the indicated mRNAs. Data were normalized by the amount of *Tbp* mRNA and are shown as means ± SD (n = 3).

(B) Representative images of SOX2 (purple) and PRRX1 (green) with DAPI (blue) in primary-cultured AL cells on day 1 (d1) and day 7 of culture (d7) are shown.

(C) Numbers of SOX2- and PRRX1-positive cells, together with cells stained with DAPI, in primary-cultured AL cells were counted and the proportion of each cell type was calculated. Data shown are means ± SD (n = 3/10 mm^2, respectively).

(D) Merged image of hormones (green) with DAPI in primary-cultured AL cells (day 7) is shown. Scale bars: 100 μm.

39. Fix the cells by adding 180 μL of the 4% PFA solution, which was brought to 20°C–25°C in advance, onto the glass surface of the glass-bottom dish for 20 min at 20°C–25°C.
40. Remove the 4% PFA solution using a micropipette, and then wash the cells three times for 5 min each with 1 mL of HBSS.
41. Permeabilize the cells by adding 180 μL of HEPES-T with 10% normal donkey serum (blocking buffer), which was brought to 20°C–25°C in advance, onto the glass surface of the glass-bottom dish for 60 min at 20°C–25°C.
42. Remove the blocking buffer using a micropipette and incubate permeabilized cells by adding 180 μL of primary antibody mixes (appropriately diluted in the blocking buffer and brought to

20°C–25°C in advance) onto the glass surface of the glass-bottom dish for 12–16 h at 20°C–25°C.

43. Remove the antibody solution using a micropipette, and wash the cells three times for 5 min each with 1 mL of HBSS.
44. Incubate permeabilized cells by adding 180 μL of secondary antibodies mixes (appropriately diluted in the blocking buffer and brought to 20°C–25°C in advance) onto the glass surface of the glass-bottom dish for 1 h at 20°C–25°C.
45. Remove the antibody solution using a micropipette, and wash the cells three times for 5 min each with 1 mL of HBSS.
46. Stain the nucleus by adding 100 μL of VECTASHIELD Mounting Medium with DAPI onto the glass surface of the glass-bottom dish for 2 h at 20°C–25°C.
47. Perform fluorescence observation and acquire immunofluorescent images using a fluorescence microscope (i.e., IXplore Pro with the imaging software cellSens Dimension System, Olympus, Tokyo, Japan).

Checking the differentiation capacity of primary-cultured AL cells

Timing: approx. 5 days

This section describes steps for the aggregate formation from primary-cultured AL cells and its immunofluorescence analysis.

48. Proceed up to step 25.
49. Remove the culture medium using a micropipette and then wash twice with 1 mL of HBSS.
50. Add 2 mL of digestion buffer B, followed by incubation at 37°C for 15 min.
51. Add 2 mL of DM to stop the trypsin reaction.
52. Transfer the cell suspension to a 15-mL conical tube using a micropipette (P1000).
53. Centrifuge at 100 × *g* for 5 min at 20°C–25°C and then remove the supernatant.
54. Add 2 mL of fresh DM and then suspend into single cells. Specifically, aspirate and expel the AL with a micropipette (dial is set to 1,000 μL) multiple times (approximately 10 times).
55. Seed at 5,000 cells/100 μL/1 well in non-adherent, U-bottom 96-well plates, and then transfer to a CO_2 incubator (5% CO_2/95% air) and culture at 37°C for 4 days.
56. Collect all aggregates from one plate into a 15-mL conical tube using a micropipette (P200).
57. Centrifuge at 100 × *g* for 5 min at 20°C–25°C and then remove the supernatant.
58. Resuspend aggregates in a small volume (up to 50 μL) of DM and keep on ice.
59. Solidify the sample into a jelly-like substance using the iPGell kit according to the manufacturer's instructions.
60. Add 1.3 mL of the 4% PFA solution and gently pipette the sample so that it peels off from the tube, and fix samples by rotating for 1 h at 4°C.
61. Remove the 4% PFA solution using a micropipette and then rinse samples with 1 mL of HBSS.
62. Remove HBSS using a micropipette and immersed samples in the 30% trehalose solution for 24 h at 4°C.
63. Embed the sample in O.C.T. Compound at –80°C before being sectioned.
64. Section the frozen sample at a thickness of 8 μm and mount on glass slides.
65. Perform immunofluorescence analysis as in step 41 through step 47.

Note: Change the volume of all solutions (i.e., antibody mixes) to 50 μL.

EXPECTED OUTCOMES

Here, we provide a cost-efficient, reproducible isolation procedure for primary adult pituitary stem/progenitor cells (APSCs) from C57BL/6J mice (8- to 12-week-old mice) without the requirement of

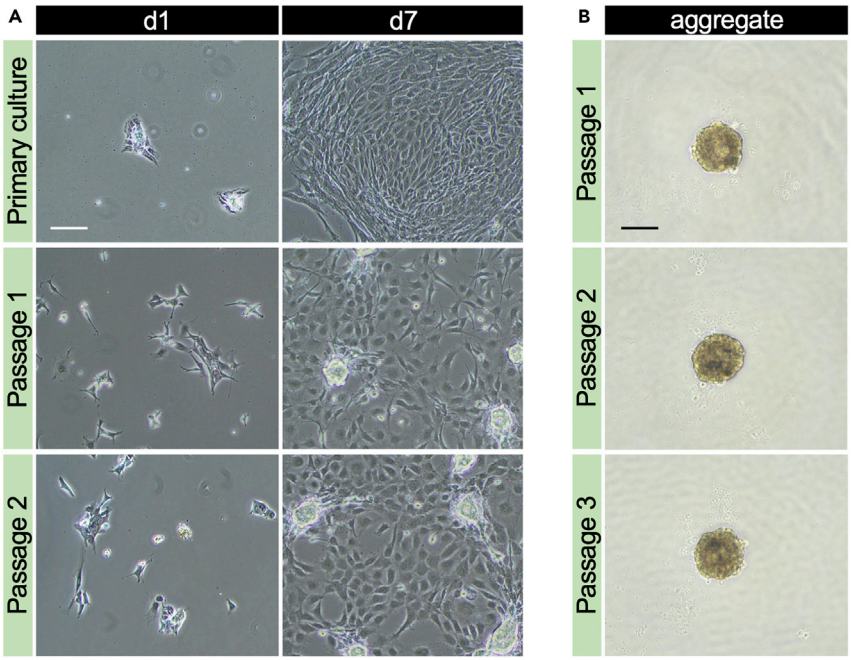

Figure 6. Passage cultures of adult pituitary stem/progenitor cells (APSCs)

(A) Primary anterior lobe cells were seeded on a glass-bottom dish and cultured for 7 days, before being dispersed by a trypsin. Dispersed cells (APSCs) were re-seeded (passage 1) on glass-bottom dishes and were cultured for a further 7 days. Images of cells on day 1 (d1) and day 7 of culture (d7) are shown. The passage culture of APSCs (passage 2) was then repeated.

(B) Images of aggregates formed from primary and passaged cells. Scale bars: 100 μm.

difficult-to-use reagents or any special equipment. This protocol has not been tested in other mouse strains.

A healthy AL will provide approximately 100,000–200,000 cells by dissociation. On the day after seeding, cell adhesion of approximately 20,000–25,000 cells was confirmed. After 7 days of culture, primary-cultured AL cells reached approximately 200,000–400,000 cells/dish. The cells grow in colonies, reaching a maximum diameter of 2 mm on average. Furthermore, these primary-cultured AL cells are capable of passaging (Figure 6A).

The isolated and cultured cells from AL showed undifferentiated properties. Therefore, we named these cell APSCs. Primary-cultured APSCs can be identified by qRT-PCR using specific primers against pituitary stem/progenitor cell markers (Figure 5A). In addition, we performed immunofluorescence analysis of SOX2 and PRRX1 as markers to confirm the purity of the APSCs (Figure 5B). In contrast, all APSCs were immuno-negative for the anterior pituitary hormone.

Most importantly, the purity of the primary APSCs was almost 100%. Moreover, three-dimensional culture of APSCs enabled differentiation into hormone-producing cells (Figure 7). This means that APSCs are suitable for studying differentiation into hormone-producing cells.

Using this protocol, we successfully isolated and described APSCs, one of the subtypes of SOX2-positive pituitary stem/progenitor cells. Classical pituitary stem/progenitor cell dissociation protocols often rely on special equipment and reagents, such as fluorescence-activated cell sorting (FACS), magnetic cell sorting (MACS), and reconstituted basement membranes isolated from mouse sarcoma cells such as Matrigel (Andoniadou et al., 2013; Chen et al., 2009; Cox et al., 2019;

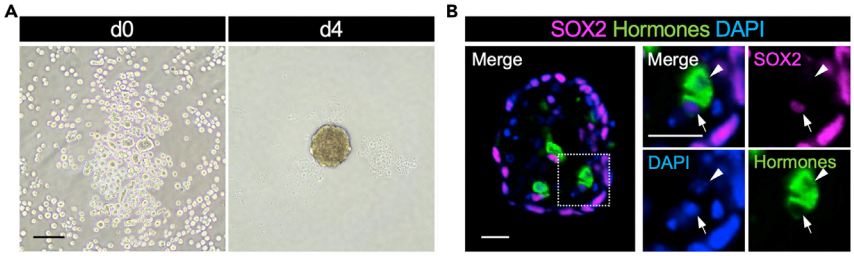

Figure 7. Differentiation capacity of primary-cultured anterior lobe (AL) cells

(A) The aggregate formed from primary-cultured AL cells by low-cell-adhesion culture. Images of cells immediately after seeding (d0) and on day 4 of culture (d4) are shown.

(B) Immunofluorescence images of SOX2 (purple) and hormones (green) with DAPI (blue) in the sliced section of the aggregate. Merged images are indicated and boxed areas are enlarged in the center and right panels. Arrows and arrowheads indicate SOX2 and hormone double positive and hormone single positive cells, respectively. Scale bars: 100 μm (A) and 20 μm (B).

Horiguchi et al., 2018). To minimize the requirement for expensive equipment and reagent costs, we chose to use 2D culture for cell isolation, which does not require special equipment or reagents.

LIMITATIONS

The number of APSCs obtained after culture varied slightly according to sex. In our experience, the number of APSCs isolated was higher in female mice due to their faster cell proliferation.

This protocol was designed to isolate primary AL cells from 8- to 12-week-old mice, and all the isolation steps were optimized for this age. Nevertheless, our protocol can be adjusted for mice of different ages. However, we cannot exclude that the number of APSCs obtained after culture may fluctuate significantly or that the expression levels of pituitary stem/progenitor cell markers may change.

We have demonstrated that APSCs can be passaged up to three times (Figure 6A). For passaging, cells should be cultured with GM instead of AM to prevent the differentiation of APSCs (See steps 49–53 for single cell dispersion). Although the cell morphology on day 7 of culture is somewhat different between primary and passage cultures, the ability to form aggregates is similar (Figure 6B). Moreover, a failure of the aggregate formation from primary AL cells prior to culture indicates that aggregates can only be established when the purity of APSCs is high (Figure 8C). Therefore, APSCs isolated in this protocol can be used for further experiments without any problems after passaging.

TROUBLESHOOTING

Problem 1

AL and IL/PL are not separated properly (step 9, Figure 8A).

Potential solution

It requires training to separate AL and IL/PL properly. If the IL/PL is disconnected, keep an eye on it and gradually peel the IL/PL off the AL.

Problem 2

Contamination of neuron-like sharp cells in primary culture (steps 22–25, Figure 8B).

Potential solution

Remove as much IL/PL as possible. In addition, if the cell density to be seeded is low, there will be many sharp cells instead of a block-like cell mass. Slowly seed the cell suspension to form a droplet.

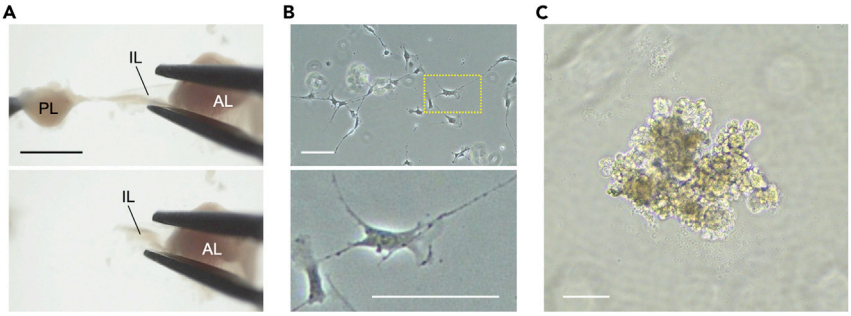

Figure 8. Images for troubleshooting

(A) Images of the intermediate lobe (IL) remaining in the anterior lobe side (AL).

(B) Images of neuron-like sharp cells in primary culture. The enlarged image is shown in the lower panel.

(C) Failure of aggregate formation using low purity adult pituitary stem/progenitor cells. Scale bars: 1 mm (A) and 100 μm (B and C).

Problem 3

Bacterial or fungal contamination (steps 22–25).

Potential solution

Adding antibiotics to the culture medium had no effect on the isolation of APSCs.

Problem 4

Hormone-producing cell contamination in isolated APSCs (steps 12–36).

Potential solution

Because APSCs can survive and proliferate even in a very harsh environment, it is possible to remove non-APSCs by intense pipetting during cell separation and rinsing during culture medium exchange.

Problem 5

No aggregates of APSCs are formed (step 55, Figure 8C).

Potential solution

For example, if the aggregate formation experiment is performed using primary AL cells immediately after the single cell suspension (day 0), no aggregates are formed. If the aggregate formation is inadequate, immunofluorescence analysis should be performed on day 1 of culture to determine the percentage of PRRX1-positive cells to assess cell quality (see, Figure 5).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Masashi Higuchi (mhiguchi@tottori-u.ac.jp).

Materials availability

This study did not involve generation of any new reagents. For specific details on availability, please refer to the key resources table.

Data and code availability

This protocol did not involve generation/analysis of datasets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, methodology, validation, investigation, writing – Original Draft, writing – Review & Editing, A.S. and M.H.; supervision and funding acquisition, M.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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