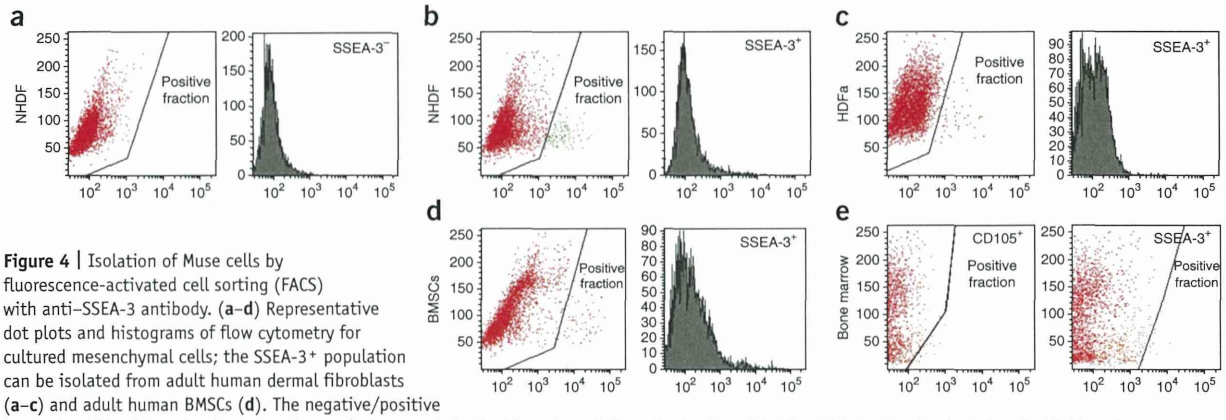

Figure 4 | Isolation of Muse cells by fluorescence-activated cell sorting (FACS) with anti-SSEA-3 antibody. (**a**–**d**) Representative dot plots and histograms of flow cytometry for cultured mesenchymal cells; the SSEA-3+ population can be isolated from adult human dermal fibroblasts (**a**–**c**) and adult human BMSCs (**d**). The negative/positive gates should be determined using each sample immunostained with anti-rat IgM antibody alone (**a**). The SSEA-3+ fraction is isolated with the primary antibody (anti-SSEA-3 antibody) and anti-rat IgM antibody (**b**–**d**). (**e**) Representative dot plots of mononuclear cells derived from fresh bone marrow aspirate. Muse cells are isolated with two primary antibodies: anti-CD105 and anti-SSEA-3.

cause cell death, further resulting in problems in FACS sorting such as cell clogging and/or a very high number of SSEA-3+ cells. Mixing or suspending cells by tapping or vortexing is not recommended for the same reason.
? TROUBLESHOOTING

9| Centrifuge the 1.8-ml microcentrifuge tube containing the sorted cells at 420*g* for 5 min, and resuspend the cells in the tube with 1 ml of FACS buffer or α-MEM containing 10% (vol/vol) FBS. Take 8.0 μl of trypan blue and transfer it into a new 1.8-ml microcentrifuge tube. Take 8.0 μl of the FACS buffer/cell mixture, transfer it into the microcentrifuge tube, mix the solution, take 8.0 μl of the solution to the hemocytometer, count the number of living cells and then proceed either to Step 10 to carry out M-cluster formation or directly to Step 19A for spontaneous differentiation on a gelatin-coated coverslip. Telomerase activity can be analyzed with 1.0 × 10^6 Muse cells (**Supplementary Fig. 1**).
▲ CRITICAL STEP Trypan blue staining is performed for counting viable cells. During FACS sorting, dead or dying cells may be sorted out by using a fluorescent viability dye such as 7-amino-actinomycin D or propidium iodide.

M-cluster formation in suspension culture TIMING 7–10 d
10| M-cluster formation from Muse cells in suspension culture can be achieved by two procedures: single-cell suspension culture (option A) or MC culture (option B). To evaluate M-cluster formation or the self-renewal property of Muse cells by cycle culture, perform a single-cell suspension culture (option A). To obtain a number of M-clusters for immunocytochemistry or transplantation experiments, according to the previous studies[39,40], perform MC culture (option B). In both cases, the surface of the bottom of each culture dish or well should be coated with poly-HEMA before starting Muse cell culture in order to avoid adhesion of the cells, because Muse cells and/or mesenchymal cells are highly adhesive. Poly-HEMA–coated culture dishes should be ready no later than the day of isolation of Muse cells by FACS.
▲ CRITICAL STEP It takes overnight for the dish to dry out, meaning that you need more than a night to make the poly-HEMA–coated dish. Do not forget to start preparing it at least 1 d before FACS sorting.
(A) Single-cell suspension culture ● TIMING 7–10 d
(i) Pick up each individual cell by a microglass pipette and transfer it to a well of a poly-HEMA–coated 96-well dish, or dispense each single cell to each well after a limiting dilution of the FACS-isolated cells. Culture the single cells with 100 μl of α-MEM containing 10% (vol/vol) FBS and 2 mM L-glutamine.
▲ CRITICAL STEP Although we have not done it, this step may be substituted by using an ACDU (automated cell deposition unit), a specific piece of equipment for the FACSAria distributed by Becton Dickinson, which enables the direct allocation of FACS-isolated cells into each well of a 96-well culture dish.
(ii) The next day, mark the empty wells or wells containing two or more cells such that these wells can be excluded from further counting and analysis.
▲ CRITICAL STEP To evaluate the technique of a limiting dilution, we recommend performing nuclear staining with Hoechst 33342 before a limiting dilution and confirming that the occurrence of two or more cells in a single well is rare.
(iii) At 7 d after starting the culture, count the number of M-clusters whose diameters are >25 μm to evaluate M-cluster formation.

(iv) To confirm the self-renewal property of Muse cells by cycle culture (**Fig. 2**), proceed from Step 11 for adherent culture. For the evaluation of Muse cells in M-clusters, proceed to Step 18. To evaluate the differentiation capacity of Muse cells, proceed from Step 19. **? TROUBLESHOOTING**

TABLE 4 | Optimized volumes of agents and medium for MC culture.

Dish	Number of cells	μ-MEM/10% (vol/vol) FBS (μl)	2.6% MC (μl)	Total (μl)
35 mm	20,000	1,330	670	2,000
60 mm	50,000	3,330	1,670	5,000

(B) MC culture ● **TIMING 7–10 d**

(i) According to **Table 4**, pour the medium containing the FACS-isolated cells, FBS, 2.6% (vol/vol) MethoCult and 200 mM L-glutamine into a 50-ml conical tube; mix well by gentle pipetting with a 1,000-μl pipette.
▲ **CRITICAL STEP** MethoCult is used to minimize the micromovement of Muse cells in order to avoid contact and cell-to-cell adhesion between the Muse cells. Because the viscosity of MethoCult is very high, it should be handled with a 1,000-μl or 200-μl pipette with the pipette tip cut out (with scissors) to enlarge the hole.

(ii) Pour an appropriate amount of the mixture of the FACS-isolated cells and MC culture medium into each well of a poly-HEMA–coated dish according to the table below.

Dish	Initial volume (μl)	Additional volume (μl)
6-well	3,000	1,300
12-well	1,250	530
24-well	700	300
48-well	400	170
96-well	130	60

▲ **CRITICAL STEP** Do not scratch the surface of the bottom of the dish with a pipette.

(iii) According to the table in Step 10B(ii), add an appropriate amount of α-MEM supplemented with 10% (vol/vol) FBS to each well of the culture dish every 3 d. At 7–10 d after starting MC culture, count the number of M-clusters whose diameter is >25 μm and calculate the proportion of M-cluster formation.

(iv) To evaluate Muse cells in M-clusters, proceed to Step 18. To confirm the differentiation capacity of Muse cells, proceed from Step 19A for spontaneous differentiation or from Step 19B for induced differentiation (**Fig. 1**). The cells treated in Step 9B(i–iv) are also applicable to transplantation experiments such as those done in previous studies[39,40].
? TROUBLESHOOTING

Adherent culture ● **TIMING 7–10 d**

11| To confirm the self-renewal property of Muse cells, cycle culture consisting of suspension culture–adherent culture–suspension culture should be performed. To set up an adherent culture, pick up each M-cluster obtained by single-cell suspension culture using a microglass pipette and directly transfer them onto a 48-well culture dish.
▲ **CRITICAL STEP** Do not dissociate an M-cluster into single cells.

12| Gently add 250 μl of α-MEM containing 10% (vol/vol) FBS into each well of a 48-well culture dish and incubate the cells in a CO_2 incubator set to 5% CO_2 and 37 °C. Change the medium every other day by discarding the old medium using gentle suction and pouring in the new medium.

13| When the number of cells expanded from an M-cluster reaches 3,000–5,000, which is usually observed at 7 d after transfer to the adherent culture, set up a water bath at 37 °C. To detach the adherent cells, prepare the following chemical agents in individual 15-ml conical tubes: 13 ml of D-PBS, 3 ml of trypsin-EDTA solution, 1.5 ml of FBS, 9 ml of α-MEM and 10 ml of D-PBS for cells cultured in a 48-well culture dish. Prewarm them in the water bath.

14| Remove the medium of the culture, add 250 μl of D-PBS to each well of a 48-well culture dish for washing and remove the D-PBS again. Apply 50 μl of trypsin-EDTA solution (see Reagents) onto each well Tilt the culture dish several times to cover the entire surface of the culture dish with trypsin-EDTA solution, and then incubate it in a CO_2 incubator set to 5% CO_2 and 37 °C for 5 min to allow the trypsin reaction to occur.

15| Confirm the detachment of the cells from the surface of the culture dish by observation under a phase-contrast microscope, and add 25 µl of FBS to each well to stop the enzyme reaction. Dissociate the cells by gentle pipetting with a 200-µl pipette, and then transfer the solution containing the dissociated cells in each well of the 48-well culture dish into individual 1.8-ml microcentrifuge tubes. Suspend the remaining cells on the surface of each well of the culture dish in 175 µl of fresh medium, transfer them into the corresponding 1.8-ml microcentrifuge tubes and centrifuge the tubes at 420*g* for 5 min.

16| Remove the supernatant, suspend the cells in 500 µl each of D-PBS for washing, centrifuge the tubes at 420*g* for 5 min and remove the supernatant again.

17| To confirm the self-renewal property of Muse cells, apply the cells to a second FACS for isolation of Muse cells (repeat Steps 2–9), followed by performing next-generation M-cluster formation (repeat Step 10A; **Fig. 2**). Alternatively, directly apply the cells to next-generation M-cluster formation (repeat Step 10A).
? TROUBLESHOOTING

Evaluation of Muse cells in M-clusters ● TIMING 1.5 h–2 d

18| Evaluate the M-clusters formed in suspension culture (either single-cell suspension culture or MC culture) by ALP reaction and immunocytochemistry.

(A) Detection of ALP activity ● TIMING 1.5 h

(i) Collect M-clusters in a 1.8-ml microcentrifuge tube, suspend them in 1 ml of saline and centrifuge the tube at 420*g* for 5 min. Discard the supernatant, add 1 ml of saline and centrifuge the tube again 420*g* for 5 min. Repeat this process (discard supernatant, add saline, centrifuge) twice.
▲ **CRITICAL STEP** Do not use D-PBS instead of saline in this step, because the phosphate in D-PBS will inactivate the ALP.

(ii) Discard the supernatant, add 100 µl of 4% (wt/vol) PFA/0.1 M PB solution and incubate the tube at 4 °C for 5 min with gentle tapping. After 5 min, add 900 µl of saline and centrifuge the tube at 420*g* for 5 min. Discard the supernatant, add 1 ml of saline and centrifuge it at 420*g* for 5 min for washing. Repeat the 1-ml wash twice.
▲ **CRITICAL STEP** Too much fixation causes a reduction in the ALP reaction. If the result of the ALP reaction is too slight to be detected, you can reduce the fixation time to 2 min or just omit the fixation procedure.

(iii) During the washing step, prepare ALP reaction solution as follows: mix 10 µl each of sodium nitrite solution and FRV-alkaline solution, leave it for 2 min at RT, add 450 µl of saline and then add 100 µl of naphthol AS-BI alkaline solution. These three solutions are all included in the Leukocyte ALP kit.

(iv) After removing the supernatant, add 200 µl of ALP reaction solution to the microcentrifuge tube containing M-clusters; incubate the tube at 37 °C for 15 min.
? TROUBLESHOOTING

(v) To stop the ALP reaction, suspend M-clusters with 800 µl of D-PBS and centrifuge the tube at 420*g* for 5 min. Discard the supernatant and add 1 ml of D-PBS. Centrifuge the tube further, and remove the supernatant to leave 100 µl of the supernatant in the microcentrifuge tube. After gentle tapping to resuspend the cells, transfer the M-clusters onto a glass microscope slide and cover them with a coverslip for observation under a light microscope.

(B) Immunocytochemistry against pluripotency markers ● TIMING 2 d

(i) Collect M-clusters into a microcentrifuge tube to evaluate the expression of pluripotency markers. Centrifuge the tube at 420*g* for 5 min. Discard the supernatant, add 100 µl of ice-cold 4% (wt/vol) PFA/0.1 M PB solution and incubate the tube at 4 °C for 30 min.
! CAUTION PFA is considered teratogenic and carcinogenic. Handle it with care.
? TROUBLESHOOTING

(ii) After fixation with 4% (wt/vol) PFA/0.1 M PB solution, suspend the M-clusters with an additional 900 µl of D-PBS and centrifuge the microcentrifuge tube at 420*g* for 5 min. Discard the supernatant, add 1 ml of D-PBS and centrifuge it at 420*g* for 5 min. Repeat the 1-ml wash twice (for a total of 3 washes).

(iii) After removing as much supernatant as possible, add an aliquot of OCT compound to the microcentrifuge tube. Soak the bottom of the tube in liquid nitrogen to freeze the M-clusters together with the OCT compound surrounding the M-clusters. Remove the frozen OCT compound containing M-clusters using a small chemical spatula or microforceps, and then transfer it into a cryomold and apply the OCT compound to fill the cryomold. After the frozen OCT compound containing M-clusters is embedded in the new OCT compound, freeze it on a dry-ice plate. Make 8-µm-thick cryosections using a cryostat.

(iv) Dry the sections using a hair dryer for 30 min, wash them with D-PBS in a Coplin jar at RT for 10 min, and then discard the solution. Incubate each section with 30 µl of blocking solution for 30 min at RT. Discard the solution and further incubate the sections with the primary antibody against Nanog, Oct3/4, Sox2, PAR4 and/or SSEA-3 diluted with 30 µl of the antibody diluent at 4 °C overnight.

(v) After incubation of the primary antibody, wash the sections three times with D-PBS for 10 min, incubate them with the corresponding secondary antibody, wash them with D-PBS and 0.1 M PB and then enclose them with a coverslip along with antifade reagent or with 0.1 M PB and glycerol (1:1). Details of the company, cat. no. and dilution of primary and secondary antibodies applied to immunocytochemical analysis are summarized in **Table 1**.

(vi) Observe the immunofluorescence signal under a fluorescence microscope.

? TROUBLESHOOTING

Evaluation of the differentiation capacity of Muse cells ● TIMING 1–3 weeks

19| The differentiation capacity of Muse cells can be evaluated under spontaneous differentiation conditions (option A) or by induction of differentiation into specific cell types (option C). When Muse cells in M-clusters are applied to induced differentiation (option C), follow option B for dissociation of M-clusters before performing induced differentiation (option C). When FACS-isolated Muse cells are applied to induced differentiation, directly follow option C.

(A) Spontaneous differentiation on a gelatin-coated coverslip ● TIMING 1–2 weeks

(i) Place a 15-mm round coverslip in the bottom of each well of a 24-well culture dish. Apply 1 ml of 0.1% (wt/vol) gelatin solution onto a 15-mm round coverslip in each well of a 24-well culture dish, and then incubate it in a CO_2 incubator set to 5% CO_2 and 37 °C for at least 45 min (but up to 3 h) to coat the coverslips with the gelatin solution. Remove the excess gelatin solution by aspiration just before use. Do not dry or wash the gelatin-coated coverslip.

(ii) At 7–10 d after starting a single-cell suspension culture or an MC culture, pick up an M-cluster with a glass micropipette and transfer it onto a gelatin-coated 15-mm round coverslip in a 24-well culture dish.

▲ **CRITICAL STEP** When transferring M-clusters picked up from MC culture, M-clusters must first be stringently washed several times by pipetting with 200 µl of α-MEM containing 10% (vol/vol) FBS using a 1,000-µl pipette in a well of a four-well culture dish. The adhesiveness of M-clusters will be lowered if this washing step is not properly performed.

(iii) Add 125–150 µl of α-MEM containing 10% (vol/vol) FBS to each well of the 24-well culture dish containing M-clusters and incubate it in a CO_2 incubator set to 5% CO_2 and 37 °C for 3 h. After this 3-h incubation, add fresh medium to make up the volume to 500 µl.

▲ **CRITICAL STEP** This procedure will help the M-clusters to adhere onto the surface of the coverslip in the bottom of each well of the 24-well culture dish. Lowering the initial volume will allow the transferred M-clusters to attach to the gelatin-coated coverslip.

▲ **CRITICAL STEP** Do not forget to add more medium after the 3-h incubation. Otherwise, the cells will dry out.

? TROUBLESHOOTING

(iv) Culture cells for up to 2 weeks, and then follow the steps from Step 20 to perform immunocytochemical analysis (Step 20A) or RT-PCR (Step 20C) to evaluate spontaneous differentiation of the single M-cluster-derived cells. The primary antibodies used are: anti-neurofilament, anti-smooth muscle actin (SMA) and/or anti-desmin for mesodermal lineage; anti-α-fetoprotein, anti-GATA4 and/or anti-cytokeratin-7 for endodermal lineage; and anti-neurofilament for ectodermal lineage cells. Information regarding these primary antibodies is provided in **Table 1**. Confirm the mRNA expression of α-fetoprotein, GATA6, microtubule-associated protein-2 (MAP-2), Nkx2.5 and β-actin by RT-PCR amplification of the relevant genes according to Step 20C. Detailed information about each primer set and annealing temperature is shown in **Table 2**.

? TROUBLESHOOTING

(B) Dissociation of M-clusters for induced differentiation of Muse cells into mesodermal-, endodermal- and ectodermal-lineage cells ● TIMING 1 h

(i) At 7–10 d after starting MC culture, pick up all the M-clusters with a glass micropipette and collect them into a microcentrifuge tube. Add 1.0 ml of D-PBS, centrifuge the tube at 420*g* for 5 min and discard the supernatant. Repeat this washing procedure twice.

(ii) To dissociate the collected M-clusters into a single cells, prepare the following chemical agents in individual 1.8-ml microcentrifuge tubes: 1 ml of D-PBS, 200 µl of trypsin-EDTA solution, 100 µl of FBS, 700 µl of α-MEM containing 10% (vol/vol) FBS and 1 ml of α-MEM containing 10% (vol/vol) FBS.

(iii) After removing the supernatant, add 1 ml of D-PBS to the tube for washing, centrifuge it at 420*g* for 5 min and remove the supernatant again. Apply 200 µl of trypsin-EDTA solution (see Reagents) to the tube and incubate it in a CO_2 incubator set to 5% CO_2 and 37 °C for 5 min to allow the trypsin reaction to take place.

(iv) Add 100 µl of FBS to stop the enzyme reaction. Dissociate the cells by gentle pipetting with a 1,000-µl pipette and dilute the mixed solution with 700 µl of fresh D-PBS. Centrifuge the tube at 420*g* for 5 min and discard the supernatant.

(C) Induced differentiation of Muse cells into mesodermal-, endodermal- and ectodermal-lineage cells ● TIMING 2–3 weeks

(i) To confirm the differentiation capacity of Muse cells into the cells representative of all three germ layers, follow the four options: Step 19C(ii–vi), induction of differentiation into osteocytes in mesodermal lineage; Step 19C(vii–ix),

induction of differentiation into adipocytes in mesodermal lineage; Step 19C(x–xi), induction of differentiation into hepatocytes in endodermal lineage and and Step 19C(xii–xvi), induction of differentiation into neural cells in ectodermal lineage.

▲ **CRITICAL STEP** To induce differentiation into mesodermal-, endodermal- and ectodermal-lineage cells, the FACS-sorted Muse cells rather than the dissociated cells derived from M-clusters are recommended for use as the source of differentiation. This is because the viability of M-cluster-derived cells is markedly decreased after dissociation.

? TROUBLESHOOTING

(ii) *Osteocyte induction*. Induce the differentiation of Muse cells into osteocytes using the human mesenchymal stem cell functional identification kit according to the manufacturer's protocols.

(iii) Prepare a working solution of fibronectin with D-PBS at a concentration of 1 μg ml^{-1} just before use, apply 500 μl of it onto a 15-mm coverslip placed at the bottom of a 24-well culture dish, and then incubate the dish at 37 °C for at least 4 h to coat the coverslip with fibronectin.

(iv) After incubation and discarding the fibronectin solution, apply 500 μl of D-PBS onto the fibronectin-coated 15-mm coverslip and discard the D-PBS for washing. Perform this washing procedure twice (for a total of three times).

(v) Plate 1.8 × 10^4 dissociated cells onto the fibronectin-coated 15-mm coverslip. Cells will reach 50–70% confluency within next 2 d. After this procedure, induce differentiation according to the manufacturer's protocol.

(vi) After 2–3 weeks, perform immunocytochemistry according to the procedure described in Step 20A. Use anti-osteocalcin antibody to detect osteocyte differentiation. Information regarding the use of this antibody is shown in **Table 1**. The timing for osteocyte induction processes (Step 19C(ii–vi)) is about 2–3 weeks.

(vii) *Adipocyte induction*. Induce the differentiation of Muse cells into adipocytes using the human mesenchymal stem cell functional identification kit according to the manufacturer's protocols.

(viii) Plate 2.3 × 10^4 dissociated cells in the manufacturer's recommended medium onto each well of a 24-well culture dish. Cells will reach 100% confluency within the next 2 d. Thereafter, follow the manufacturer's protocol.

(ix) After 2–3 weeks, evaluate adipocyte differentiation by observing the morphology of the induced cells under phase-contrast microscopy to detect lipid droplets inside the cytoplasm and perform oil red staining according to the manufacturer's recommended protocol. The timing for adipocyte induction processes (Step 19C(vii–ix)) is about 2–3 weeks.

(x) *Hepatocyte induction*. Plate 1.6 × 10^5 cells onto a collagen-coated 35-mm culture dish and culture them with 2 ml of DMEM supplemented with 200 μl of FBS (final concentration: 10% (vol/vol)), 200 μl of ITS (10×), 2 μl of 10 μg ml^{-1} dexamethasone (final: 10 nM), 2 μl of 100 μg ml^{-1} HGF (final: 100 ng ml^{-1}) and 4 μl of 25 μg ml^{-1} FGF-4 (final: 50 ng ml^{-1}).

(xi) Fourteen days after starting the induction into hepatocytes, follow Step 20A or 20C in order to perform immunocytochemical analysis or RT-PCR. Use anti-α-fetoprotein antibody to confirm the differentiation of Muse cells into hepatocytes (**Table 1**). Confirm the expression of mRNA for human α-fetoprotein and β-actin by RT-PCR. The specific primer sets and annealing temperatures are available in **Table 2**. The timing for hepatocyte induction processes (Step 19C(x–xi)) is ~2 weeks.

(xii) *Neural induction*. Prepare a poly-HEMA–coated dish before neural induction. Perform poly-HEMA coating for a 35-mm culture dish as described above.

(xiii) Mix the following chemical agents for 10 ml of the neural precursor cell culture medium: 9.58 ml of Neurobasal medium, 200 μl B-27 supplement (50×), 100 μl of kanamycin, 100 μl of 200 mM L-glutamine (final concentration: 2 mM), 10 μl of 30 μg ml^{-1} bFGF (final: 30 ng ml^{-1}) and 10 μl of 30 μg ml^{-1} EGF (final: 30 ng ml^{-1}). This neural precursor cell culture medium should be prepared each time just before use.

(xiv) Plate 1.0 × 10^5 Muse cells onto a poly-HEMA–coated 35-mm culture dish and culture them with 2 ml of the neural cell culture medium for up to 7 d in order to induce the differentiation into neural precursor cells that form the specific cell aggregates called spheres. To confirm the differentiation of Muse cells into neural precursor cells, follow Step 20B to perform immunocytochemical analysis.

(xv) Prepare the PLL-coated coverslips before starting the differentiation assay into neurons. PLL is provided as 0.1% (wt/vol) stock solution, which should be diluted 10 times with DDW to give the working solution. It should be prepared each time before use. In order to coat a 25-mm coverslip on a 35-mm culture dish, apply 1.0 ml of PLL working solution onto a 25-mm coverslip and incubate it in a CO_2 incubator set to 5% CO_2 and 37 °C for at least 4 h. After incubation, discard the PLL working solution by aspiration and add 2 ml of DDW. Repeat this washing procedure up to three times. Air-dry the coverslip in a tissue culture hood without air flow and with the UV lamp switched off overnight.

■ **PAUSE POINT** After air drying, store coated coverslips at 4 °C. The effect of the coating is valid for up to 6 months after coating.

(xvi) Remove the medium from the culture dish and add 2 ml of α-MEM for washing. Repeat this washing step twice. After discarding the medium again, plate cells onto a PLL-coated 25-mm coverslip on a 35-mm dish and culture the cells with 2 ml of α-MEM containing 40 μl of FBS (final concentration: 2% (vol/vol)), 1.7 μl of 30 μg ml^{-1} bFGF (final: 25 ng ml^{-1}) and 2 μl of 25 μg ml^{-1} BDNF (final: 25 ng ml^{-1}) to induce the differentiation of the cultured cells into neurons. Ten days after the medium is changed for differentiation, follow Step 20A to perform immunocytochemical analysis. Anti-MAP2 antibody is used to confirm the differentiation of Muse cells into neuronal cells (**Table 1**). The timing for neural induction processes (Step 19C(xii–xvi)) is ~3 weeks.

Evaluation of cell differentiation ● TIMING 5 h–2 d

20| To evaluate cell differentiation, perform immunocytochemical analysis (options A and B) and RT-PCR (option C).

(A) Immunocytochemical analysis of the differentiated cells ● TIMING 2 d

(i) After discarding the culture medium, apply the same amount of D-PBS and discard it by aspiration. Repeat this washing step three times.

! CAUTION Do not wash cells that have undergone neural induction. Induced neural cells will easily detach from the coverslip; therefore, 4% (wt/vol) PFA/0.1 M PB solution must be directly added soon after removing the culture medium.

(ii) Fix the cells on a coverslip with 4% (wt/vol) PFA/0.1 M PB solution for 20 min at RT. The amount of the fixative is 1 ml for a 15-mm coverslip in a well of a 24-well culture dish or 2 ml for a 25-mm coverslip in a 35-mm culture dish.

! CAUTION PFA is considered teratogenic and carcinogenic. Handle it with care.

(iii) After discarding the fixative, apply D-PBS at RT to the cells on the coverslip, incubate the cells with D-PBS for 10 min and discard the D-PBS. Repeat this washing step three times. Incubate each coverslip with 30 μl of the blocking solution for 30 min at RT, discard the solution, and then further incubate it overnight with the primary antibodies listed in Steps 19A(iv), 19C(vi), 19C(xi) and 19C(xvi) diluted with an appropriate amount of the antibody diluent at 4 °C. After incubation of the primary antibody, wash the coverslip with D-PBS, incubate it with the corresponding secondary antibody for 2 h at RT and wash it with D-PBS and 0.1 M PB. Thereafter, pick up the coverslip and place it on a glass slide with antifade reagent or 0.1 M PB and glycerol (1:1) to enclose it.

(iv) Observe the immunofluorescence signal under a fluorescence microscope.

? TROUBLESHOOTING

(B) Immunocytochemical analysis of the sphere cells ● TIMING 2 d

(i) After neural precursor induction by the treatment with bFGF and EGF, the floating cells form the cell aggregates called spheres. As these spheres can be handled like the M-clusters formed from Muse cells, follow Step 18B to perform immunocytochemical analysis. In this case, instead of antibodies against pluripotency markers, use the primary antibodies: anti-nestin, anti-musashi-1 and anti-NeuroD to confirm the differentiation of Muse cells into neural precursor cells. Information regarding these antibodies is shown in **Table 1**.

(C) RT-PCR ● TIMING 5 h

(i) Collect and transfer more than 10,000 differentiated cells into a microcentrifuge tube. Extract and purify total RNA using Nucleospin RNA XS according to the manufacturer's instructions. Generate the first-strand cDNA by reverse transcription of mRNA using the SuperScript VILO cDNA synthesis kit according to the manufacturer's protocol.

(ii) Amplify the cDNA of genes encoding β-actin, α-fetoprotein, GATA6, MAP-2, Nkx2.5, brachyury and albumin using the specific primer sets shown in **Table 2** in a 50-μl volume. Use human whole embryo as the positive controls for GATA6, MAP-2, Nkx2.5 and albumin, and use human fetal liver as the positive control for α-fetoprotein. The PCR conditions with hot start are as follows:

Cycle number	Denaturing	Annealing	Elongation
1	95°C, 5 min		
2–36	95°C, 1min	1 min (see **Table 2**)	72 °C, 1 min
37			72 °C, 7 min

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 5**.

TABLE 5 | *Troubleshooting table.*

Step	Problem	Possible reason	Solution
Reagent Setup	poly-HEMA was not dissolved	Use of improper solvent	When making poly-HEMA solution, make 95% (vol/vol) ethanol first and then apply poly-HEMA powder to it. Do not try to dissolve poly-HEMA powder with 100% ethanol so as to add DDW to make 95% ethanol, because poly-HEMA powder cannot be dissolved in 100% ethanol
		Improper dissolving method	When dissolving poly-HEMA powder, pour 95% ethanol into a 50-ml conical tube first and then apply an appropriate amount of poly-HEMA powder. If poly-HEMA powder is applied before pouring 95% ethanol, it will be aggregated and will not easily dissolve
		Insufficient dissolving time for poly-HEMA	Try to dissolve poly-HEMA powder at 37 °C for several hours. Also, bear in mind that it needs more than an overnight to dry out a dish coated with poly-HEMA solution. Start preparing the poly-HEMA-coated dish the day before starting the cell cluster formation
1A(vi)	No proliferation of cells after thawing	The supplier's recommended medium was not used in the initial culture after thawing	When cells are purchased from a supplier, they are generally cryopreserved. After thawing the cells, initial culture should be started with the medium that is recommended by the supplier, as that medium is optimized for the best growth conditions. In addition, the warranty will not be valid if the recommended medium is not used. Cells must be maintained and expanded with the medium that was provided or recommended at least by 2–3 passages
		The condition of freezing cells was not appropriate	Do not store the frozen cell-containing cryotube in a deep freezer set at −80 °C, because cells will degrade and lose viability if they are stored at −80 °C for a long time. The next day after freezing the cells in a deep freezer, the cryotube should be transferred to a cryogenic refrigerator that contains an appropriate amount of liquid nitrogen. Also, cells frozen with too little cryopreservative medium (Cell Banker) do not proliferate after thawing. When making cryopreservation stock, cells at 90% confluency should be suspended in 1 ml of Cell Banker and stored
	Too-slow proliferation of cells	Lack of L-glutamine	Supply L-glutamine as 2 mM for the working concentration
		Inappropriate procedure for passaging cells	Check the following issues: days needed to reach 90% confluency after passaging cells (usually 2–3 d after replating); morphology, which is usually long thin cell bodies with a large nucleus referred to as 'fibroblast-like morphology'. If too many days are required for cells to reach 90% confluency or if cells are polygonal or round shapes, the procedure for passaging the cells might be inappropriate. Passage the cells to 1:2 when they reach 90% confluency and use those whose passage number is from 4 to 11
		Use of poor serum	Use another serum for culturing. More than 20 types of sera from different products or in different lots should be evaluated in a lot check. When too few sera are evaluated, the appropriate serum for cell culturing will not be obtained

(continued)

TABLE 5 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
	Poor or deformed morphology, even within passage 11	Inappropriate procedure for passaging cells	Perform passaging when cells reach 90% confluency. When cells at 100% confluency or at overconfluency are subjected to passaging, cells lose proliferative activity and their morphology becomes deformed (polygonal or round shapes); these cells will not further proliferate
1A(x)	Insufficient detachment when passaging	Deactivation of trypsin	When cells are firmly adhered to the dish and not detaching well, incubate the cells with trypsin solution for 1 or 2 more minutes or further add 1 ml of trypsin solution to detach the cells from the bottom of the dish. If the cells further cannot be detached, nevertheless, trypsin solution is considered inactivated. In such a case, replace the trypsin with the new one. Note that trypsin digestion should be performed at 37 °C
8	Pausing of FACS sorting because of cells clogging the system	Cells not strained	Pass the cells through a 100-μm-pore cell strainer just before applying them to FACS sorting. Mesenchymal cells tend to adhere to each other to make aggregates that will clog the sample tubes of the FACS machine
		Too many dead cells	Gently handle cells to avoid cell death in each step. Dead cells have high viscosity and adhesiveness so that they easily make cell aggregates even if they have already passed through a 100-μm-pore cell strainer. It is recommended to use a microtube mixer for mixing the solution containing cells instead of performing gentle pipetting
	Too few SSEA-3$^+$ cells	Incomplete mixing of the staining solution	Mix well the cell-containing FACS buffer by gentle pipetting with a 1,000-μl pipette or by a microtube mixer every 10 min when incubating cells with antibodies for FACS staining
		Too-high cell concentration of cells in immunostaining	A cell concentration of more than 1.0 × 106 cells per 100 μl of FACS buffer will result in insufficient labeling of SSEA-3 cells. Suspend the cells for immunostaining in a concentration less than 1.0 x 106 cells per 100 μl
		Use of non-recommended antibody	Use anti–SSEA-3 antibody provided by Millipore. The SSEA-3 antibodies supplied by other companies will not give stable results
		Application of cells passaged too many times	Use cultured cells whose number of passaging is between 4 and 10. Use of cells whose passaging number is <4 will give unstable results, and cells beyond passage 10 may result in a much lower frequency of SSEA-3$^+$ cells
		Inappropriate procedure for passaging cells	The procedure of passaging cells greatly influences the number of SSEA-3$^+$ cells. Ensure that cells are passaged at 90% confluency; check the time of trypsin incubation, (usually 5 min); check the freezing and thawing procedure
		Inefficiency of serum	Inefficient serum might give too few SSEA-3$^+$ cells. We have not experienced this situation thus far. Before considering the inefficiency of serum, other possibilities should be assessed
	Too many (>10%) SSEA-3$^+$ cells	Death of the majority of cells	Gently handle cells to avoid cell death in each step. Dead cells nonspecifically bind to secondary antibodies, so that large numbers of cells causes high background staining, which may lead to false positive in FACS. Use a microtube mixer for mixing instead of gentle pipetting

(continued)

TABLE 5 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
		Excessive incubation of cells with the secondary antibody	Incubate cells with secondary antibody for no longer than 1 h. Too-long incubation of the cells with the secondary antibody results in too-high background staining
	Too many SSEA-3$^+$ cells when using secondary antibodies other than those labeled with FITC	Using a secondary antibody labeled with a fluorescent dye such as APC, Alexa Fluor 647 or DyLight649	Use the FITC-labeled secondary antibody for FACS sorting of SSEA-3$^+$ cells. When the secondary antibody for anti–SSEA-3 antibody is conjugated to a fluorescent dye that is excited by red laser (wavelength: 633 nm), the percentage of SSEA-3$^+$ cells will be higher than that expected
	Variable percentage of SSEA-3$^+$ cells	FACS sorting just after thawing the frozen cells	Do not use the cells just after thawing from cryopreservation for FACS sorting. Even if the number of passaging is between 4 and 10, use of cells that are just thawed before FACS sorting gives unstable results in terms of the number of SSEA-3$^+$ cells
10A(iv), 10B(iv)	No or insufficient production of cell clusters	Improper cell condition caused by inappropriate cell culturing	Check the following issues: days needed to reach 90% confluency after passaging cells (usually 2–3 d after re-plating); morphology (usually fibroblast-like morphology). If too many days are required for cells to reach 90% confluency or if cultured cells are polygonal or round, the procedure of passaging the cells might be inappropriate. Passage cells to 1:2 when they reach 90% confluency and use the cells from P4 to P11
		Cell damage in FACS sorting	Use a 100-μm-pore nozzle for FACS sorting, and sort the cells at a flow rate less than 8,000 events. Even if the number of dead cells (determined by trypan blue staining) is low, it does not indicate that there is no cell damage during FACS sorting
		Use of improper medium	Use α-MEM for cluster formation for NHDFs, HDFas, and BMSCs. If you apply other cell types for FACS sorting of Muse cells, you will need to optimize this
	No or insufficient production of cell clusters after thawing SSEA-3$^+$ cells	Improper thawing method	Do not warm the frozen cells until they are completely melted. Use the warm medium to melt the frozen cells; alternatively, cold medium or medium at room temperature can be used depending on the cell types
		Improper freezing method of cells	Recover the condition of FACS-sorted cells by suspension culture for 16 h before freezing cells for storage. If it is not performed, cluster formation will be markedly decreased
	No or insufficient production of cell clusters after adherent culture	Reaching 100% confluency during adherent culture	Do not apply the cells that reach 100% confluency in culture to FACS sorting
	Emergence of matrix- or debris-like products around the cell clusters	Normal phenomenon	This happens normally and does not require intervention
10B(iv)	No or insufficient production of cell clusters	Impurities in MC may exert a negative effect	Use another lot of MC. Because MC is a chemical compound derived from cellulose that is extracted usually from the seaweed, it contains impurities that sometimes cause a negative effect on cluster formation

(continued)

TABLE 5 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
		Improper composition of MC	Apply and mix the appropriate amounts of chemical reagents for MC culture according to **Table 4**. Higher concentration of MC raises the stiffness of the culture medium to physically inhibit the growth of cell cluster. Lower concentration of the MC will raise the mobility of cells to cause cell-to-cell contact between neighboring cells and may cause cell aggregation, which is an inappropriate condition for cluster formation. To confirm the self-renewal property of Muse cells, single cells must form a cell cluster
17	Decreasing percentages of SSEA-3$^+$ cells (<50%) when FACS sorting after Muse cell expansion in adherent culture	Overexpansion of cluster-derived cells in adherent culture	Do not expand the cells from a single cell cluster to over 5,000. The percentage of SSEA-3$^+$ cells is usually 40–50% when the cell number derived from a single cell cluster is 3,000–5,000; however, it will decrease by <10% when the total cell number reaches 50,000–100,000
18A(iv)	No or too low level reaction	Use of PBS instead of saline	Use saline for washing cells and use PBS for stopping the ALP reaction. The ALP reaction will be decreased or even inhibited when PBS is used instead of saline
		Overfixation	Weaken the condition of fixation. For example, decrease the concentration of paraformaldehyde and/or the incubation time, or just perform ALP reaction without fixation. ALP reaction is possible without fixation
		Temperature is too low	Perform the ALP reaction at 37 °C. Room temperature is not easy to control, and the ALP reaction at room temperature will be unstable
18B(i)	Some of the cell clusters remain floating after centrifugation	Contamination of MC	Perform pipetting gently but thoroughly to eliminate MC because the precipitation of cells by centrifugation will be interfered with by MC residue
18B(vi)	No cell cluster detected	Discarding the cells during suctioning of the supernatant after centrifugation	Be careful not to suction the cell pellet
		Improper discrimination between cells and dusts	Perform counterstaining of the nuclei with DAPI to discriminate cells from dust
19A(iii)	The cell cluster does not firmly adhere to a gelatin-coated coverslip	Medium was not sequentially supplemented	For the adhesion of a cell cluster on the surface of a coverslip, start cell cluster culturing with a small amount of medium (125–150 µl) and add medium up to 500 µl, 3 h after starting culturing. If this is not done, cells in the cell cluster will dry out
		Contamination of MC	Perform pipetting gently but thoroughly to eliminate MC because the adhesion of a cell cluster will be interfered with by the residue of MC
19A(iv)	The specific cell type cannot be detected in cultured cells on a gelatin-coated coverslip	Contamination of MC	Do not culture cells on a gelatin-coated coverslip until they reach 100% confluency or specific cell types will be lost
19C(i)	Detachment of cells from the bottom of the dish during induction of differentiation	Rough handling of cells	Gently handle the cells. Some types of differentiated cells such as neurons will be easily detached from the bottom of the dish. But it is not recommended to use substrate-coated dishes for cell adhesion because it can influence cell differentiation

(continued)