these cells is also described here (Step 1B). Next, we summarize the protocol for isolating Muse cells from mesenchymal cell populations by FACS (Steps 2–9). After isolation, Muse cells can be cultured under suspension (Step 10) and adherent (Steps 11–17) culture conditions. Muse cells in the adherent culture have a cell shape similar to that of regular BMSCs or dermal fibroblasts, such that they cannot be distinguished from other mesenchymal cells by their morphology. However, once they have been isolated by FACS and cultured in a suspension, Muse cells form a cluster that is similar to an ES cell–derived embryoid body. Here, we name this cell cluster the M-cluster (Muse cell–derived cell cluster). The protocol for evaluating Muse cells in the M-cluster is described in Step 18. M-clusters contain cells positive for alkaline phosphatase (ALP) reaction (Step 18A) and for pluripotency markers Sox2, Oct3/4 and Nanog, in addition to SSEA-3 (Step 18B). These M-clusters reach their maximum size by days 7–10 in a suspension culture and then cease growing; however, when they are transferred to an adherent culture they start to proliferate again. Such a cycle culture, comprising suspension culture–adherent culture–suspension culture, confirms the self-renewal property of Muse cells (**Fig. 2**). Triploblastic differentiation of Muse cells can be examined in either spontaneous differentiation (Step 19A) or induced differentiation conditions (Step 19B). In the former method, after transfer of a single M-cluster onto a gelatin-coated coverslip, the cells expanding from the adhered M-cluster spontaneously differentiate into representatives of all three germ layers. In the latter, Muse cells collected by FACS are treated with certain sets of cytokines and chemical reagents to differentiate into osteocytes, adipocytes, neural cells or hepatocytes. The protocols for detecting the expression of cell differentiation markers by immunocytochemistry and reverse transcription PCR (RT-PCR) are also described (Step 20). These procedures clearly confirm that Muse cells fulfill the requirements of stem cells in terms of their capacity for both triploblastic differentiation and self-renewal.

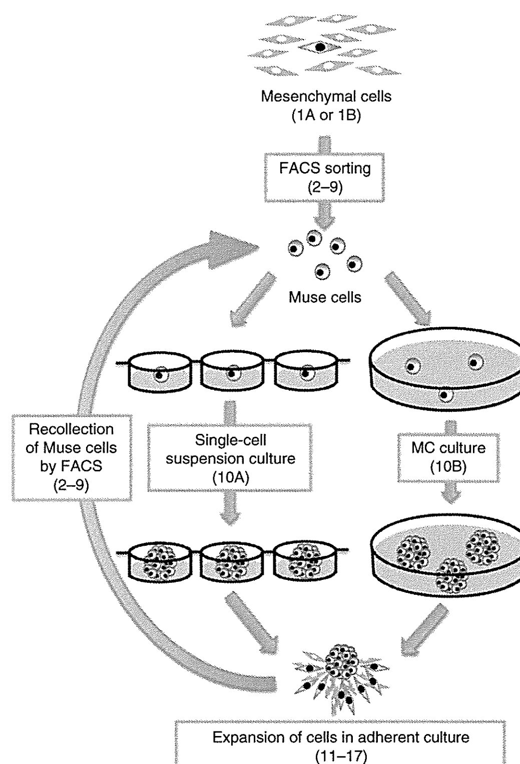

Figure 2 | Schematic diagram of the self-renewal of Muse cells. Numbers in parentheses correspond to Step numbers in the PROCEDURE.

MATERIALS

REAGENTS

- Normal human dermal fibroblasts derived from adult skin (NHDFs; Lonza, cat. no. CC-2511)
- Human dermal fibroblasts, adult (HDFas; ScienCell, cat. no. 2320)
- BMSCs (Lonza, cat. no. PT-2501)
- Normal human whole bone marrow (ALLCELLS, cat. no. ABM001, ABM003)
- Minimum essential medium Eagle (α-MEM; Sigma-Aldrich, cat. no. M4526)
- Dulbecco's modified Eagle medium, high glucose 1× (DMEM; Invitrogen, cat. no. 11965-092)
- Dulbecco's PBS (D-PBS; Wako, cat. no. 045-29795)
- FBS (HyClone, cat. nos. SH30370.03, SH30088.03 or SH30406.02; or Invitrogen, cat. no. 26140-079) ▲ **CRITICAL** A lot check should be performed before carrying out full-scale culturing. A method for lot checking is also described in this article.
- Kanamycin sulfate, 100× (Invitrogen, cat. no. 15160-054)
- L-Glutamine (Sigma-Aldrich, cat. no. G8540)
- Trypsin-EDTA solution (0.25% (wt/vol) trypsin/1 mM EDTA-4Na (1×), liquid; Invitrogen, cat. no. 25200-072)
- Cryopreservative medium, Cell Banker 1 Plus (Juji-Filed, cat. no. BLC-1P)
- Trypan blue (GIBCO, cat. no. 15250-061)
- BSA (Nacalai Tesque, cat. no. 01860-65)
- Disodium EDTA (Nacalai Tesque, cat. no. 15111-45)
- BlockAce (DS Pharma Biomedical, cat. no. UK-B40)
- Lymphoprep tube (Axis-Shield, cat. no. 1019818)
- Red blood cell lysis solution (Miltenyi Biotech, cat. no. 130-094-183)
- Ethanol (99.5% (vol/vol); Wako, cat. no. 057-00451)
- Biofreezing vessel, BICELL (Nihon Freezer)
- Anti–SSEA-3 rat IgM antibody, clone MC-631 (Millipore, cat. no. MAB4303) ▲ **CRITICAL** Anti–SSEA-3 antibody is available from several other companies, but Millipore's antibody is strongly recommended for isolation of Muse cells. When SSEA-3 antibodies from other companies are used, a proper result cannot be guaranteed.
- Purified anti-CD105 mouse antibody (BD Pharmingen, cat. no. 555690)
- FITC–affinity-purified goat anti-rat IgM antibody, μ-chain specific (min X Hu, Bov, Hrs, Sr Prot; Jackson ImmunoResearch Laboratories, cat. no. 112-095-075)
- Dylight649–affinity-purified goat anti-mouse IgG antibody, Fcγ fragment specific (min X Hu, Bov, Hrs, Sr Prot; Jackson ImmunoResearch Laboratories, cat. no. 115-495-071)
- Human serum (BioPredict, cat. no. SER019)
- TRAPEZE XL telomerase detection kit (Millipore, cat. no. S7707)
- Methylcellulose MethoCult H4100 (StemCell Technologies, cat. no. 04100)
- Poly (2-hydroxethyl methacrylate) (poly-HEMA; Sigma-Aldrich, cat. no. P3932)
- Leukocyte ALP kit, based on naphthol AS-BI and Fast Red violet LB (Sigma-Aldrich, cat. no. 86R)
- Sodium chloride (Nacalai Tesque, cat. no. 31320-05)
- Regular micro slide, glass (Matsunami, cat. no. S1225)
- Coverslips (18 mm × 32 mm; Matsunami, cat. no. C018321)

TABLE 1 | Primary and secondary antibodies.

Use	Primary or secondary	Antigen	Host species	Polyclonal/ monoclonal, clone	Company or provider	Cat. no.	Concentration	Conjugates	Notes
FACS sorting	Primary	SSEA-3	Rat	Monoclonal, MC-631, IgM	Millipore	MAB4303	1:50		Use this Millipore antibody for FACS, or the frequency of SSEA-3$^+$ cells will fluctuate
FACS sorting	Primary	CD105	Mouse	Monoclonal, 266, IgG1	Becton Dickinson	555690	1:50		Mesenchymal cell marker
FACS sorting	Secondary	Rat IgM	Goat	Polyclonal	Jackson Immuno-Research Laboratories	112-095-075	1:100	FITC	μ-chain–specific FITC-labeled antibody; minimum cross-reaction to human, bovine and horse serum proteins
FACS sorting	Secondary	Mouse IgG	Goat	Polyclonal	Jackson Immuno-Research Laboratories	115-495-071	1:100	DyLight649	Fcγ-specific, DyLight649-labeled antibody; minimum cross-reaction to human, bovine and horse serum proteins
Immunocytochemistry	Primary	Nanog	Rabbit	Polyclonal	Chemicon	AB5731	1:500		Pluripotency marker
Immunocytochemistry	Primary	Oct3/4	Rabbit	Polyclonal	Gift from H. Hamada		1:800		Pluripotency marker
Immunocytochemistry	Primary	Sox2	Rabbit	Polyclonal	Abcam	ab59776	1:1,000		Pluripotency marker
Immunocytochemistry	Primary	PAR4	Mouse	Monoclonal, A-10, IgG2a	Santa Cruz Biotechnology	sc-1666	1:100		Pluripotency marker
Immunocytochemistry	Primary	Neurofilament	Rabbit	Polyclonal	Chemicon	AB1987	1:200		Neuronal (ectodermal) marker
Immunocytochemistry	Primary	Smooth muscle actin	Mouse	Monoclonal, 1A4	LabVision	MS-113-P0	1:100		Smooth muscle cell (mesodermal) marker
Immunocytochemistry	Primary	α-fetoprotein	Rabbit	Polyclonal	DAKO	N1501	1:100		Hepatocyte (endodermal) marker
Immunocytochemistry	Primary	Cytokeratin-7	Mouse	Monoclonal, RCK105, IgG1	Chemicon	MAB3226	1:100		Hepatic (endodermal) marker
Immunocytochemistry	Primary	Desmin	Mouse	Monoclonal, RD301, IgG2b	BD Biosciences	550626	1:100		Muscle cell (mesodermal) marker
Immunocytochemistry	Primary	Osteocalcin	Mouse	Monoclonal, 190125, IgG1	R&D Systems	MAB1419	1:10		Osteocyte (mesodermal) marker
Immunocytochemistry	Primary	Nestin	Mouse	Monoclonal, Rat-401, IgG1	Millipore	MAB353	1:100		Neural progenitor (ectodermal) marker
Immunocytochemistry	Primary	Musashi	Rabbit	Polyclonal	Millipore	AB5977	1:200		Neural progenitor (ectodermal) marker

(continued)

TABLE 1 | Primary and secondary antibodies (continued).

Use	Primary or secondary	Antigen	Host species	Polyclonal/ monoclonal, clone	Company or provider	Cat. no.	Concentration	Conjugates	Notes
Immunocytochemistry	Primary	NeuroD	Rabbit	Polyclonal	Millipore	AB15580	1:200		Neural progenitor (ectodermal) marker
Immunocytochemistry	Secondary	Rabbit IgG	Donkey	Polyclonal	Invitrogen	A-21206	1:500	Alexa488	Detection of primary antibodies raised in rabbit for immunocytochemistry
Immunocytochemistry	Secondary	Rabbit IgG	Donkey	Polyclonal	Invitrogen	A-10042	1:500	Alexa568	Detection of primary antibodies raised in rabbit for immunocytochemistry
Immunocytochemistry	Secondary	Mouse IgG	Donkey	Polyclonal	Invitrogen	A-21202	1:500	Alexa488	Detection of primary antibodies raised in mouse for immunocytochemistry
Immunocytochemistry	Secondary	Mouse IgG	Donkey	Polyclonal	Invitrogen	A-10037	1:500	Alexa568	Detection of primary antibodies raised in mouse for immunocytochemistry
Immunocytochemistry	Secondary	Rat IgM	Goat	Polyclonal	Jackson ImmunoResearch Laboratories	112-095-075	1:100	FITC	Detection of the SSEA-3 primary antibody

- Paraformaldehyde (PFA; Merck, cat. no. 1040051000) **! CAUTION** This chemical is provided as powder. When you are weighing this reagent, avoid inhalation because of its teratogenicity and carcinogenicity.
- Sodium hydroxide solution (2 N; Wako, cat. no. 194-05631)
- Disodium hydrogenphosphate 12-water (Nacalai Tesque, cat. no. 31723-35)
- Sodium dihydrogenphosphate dihydrate (Nacalai Tesque, cat. no. 31718-15)
- OCT compound (Sakura Finetek, cat. no. TissueTek 4583)
- Liquid nitrogen
- Dry ice
- Triton X-100 (Sigma-Aldrich, cat. no. X100)
- Glycerol (Nacalai Tesque, cat. no. 17018-25)
- Antibodies for immunocytochemical analysis (described in **Table 1**)
- Gelatin (Sigma-Aldrich, cat. no. G1890)
- Neurobasal medium (Invitrogen, cat. no. 21103-049)
- B-27 supplement, 50× (Invitrogen, cat. no. 17504044)
- Basic fibroblast growth factor (bFGF; PeproTech, cat. no. 100-18B)
- Epidermal growth factor (EGF; PeproTech, cat. no. AF-100-15)
- Poly-L-lysine (PLL) solution (0.1% (wt/vol) in H_2O; Sigma-Aldrich, cat. no. P8920)
- Brain-derived neurotrophic factor (BDNF; PeproTech, cat. no. 450-02)
- Human mesenchymal stem cell functional identification kit (R&D Systems, cat. no. SC006)
- Insulin-transferrin-selenium (ITS; Gibco, cat. no. 51500)
- Dexamethasone (Sigma-Aldrich, cat. no. D4902)
- Hepatocyte growth factor (HGF; PeproTech, cat. no. 100-39)
- FGF-4 (R&D Systems, cat. no. 235-F4)
- Human fibronectin (R&D Systems, cat. no. 1918-FN)
- Nucleospin RNA XS (Takara, cat. no. U09025)
- RNeasy mini kit (Qiagen, cat. no. 74104)
- SuperScript VILO cDNA synthesis kit (Invitrogen, cat. no. 11754-050)
- Ex Taq (Takara, cat. no. RR001A)
- Primer sequences for RT-PCR (**Table 2**)
- Human whole fetus poly A^+ RNA (Clontech, cat. no. 636185)
- Human fetal liver total RNA (Clontech, cat. no. 636540)
- Stock solutions (see Reagent Setup)
- Water, double distilled (DDW)

EQUIPMENT

- Microcentrifuge tubes (1.8 ml; BM Bio, cat. no. BM-15)
- Polypropylene conical tubes (15 ml; BD Falcon, cat. no. 352096)
- Polypropylene conical tube (50 ml; BD Falcon, cat. no. 352070)
- Tissue culture dish (35 mm; Thermo Scientific, Nalge Nunc, cat. no. 153066)
- Tissue culture dish (60 mm; Thermo Scientific, Nalge Nunc, cat. no. 150288)
- Tissue culture dish (90 mm; Thermo Scientific, Nalge Nunc, cat. no. 150350)
- Multidish (four well; Thermo Scientific, Nalge Nunc, cat. no. 176740)
- Multidish (six well; Thermo Scientific, Nalge Nunc, cat. no. 140675)
- Multidish (12 well; Thermo Scientific, Nalge Nunc, cat. no. 150628)
- Multidish (24 well; Thermo Scientific, Nalge Nunc, cat. no. 142475)
- Multidish (48 well; Thermo Scientific, Nalge Nunc, cat. no. 150684)
- Multidish (96 well; Thermo Scientific, Nalge Nunc, cat. no. 167008)
- Round coverslip (15 mm; Matsunami, cat. no. C015001)
- Round coverslip (25 mm; Matsunami, cat. no. C025001)
- Serological disposable pipette (5 ml; BD Falcon, cat. no. 357543)
- Serological disposable pipette (10 ml; BD Falcon, cat. no. 357551)
- Serological disposable pipette (25 ml; BD Falcon, cat. no. 357525)
- Sterile polypropylene centrifuge tube (15 ml; BD Falcon, cat. no. 352096)
- Sterile polypropylene centrifuge tube (50 ml; BD Falcon, cat. no. 352070)
- Pipette (2 µl; Gilson, cat. no. MSF144801)
- Pipette (20 µl; Gilson, cat. no. MSF123600)
- Pipette (200 µl; Gilson, cat. no. MSF123601)
- Pipette (1,000 µl; Gilson, cat. no. MSF123602)
- Filtered pipette tips (10 µl; BioPoint Scientific, cat. no. 311-4050)
- Filtered pipette tips (20 µl; BioPoint Scientific, cat. no. 341-4050)
- Filtered pipette tips (200 µl; BioPoint Scientific, cat. no. 351-4050)

TABLE 2 | Primer information for RT-PCR.

Gene (symbol)	Forward primer sequence	Reverse primer sequence	Annealing temperature (°C)	Note
Human β-actin (*ACTB*)	5′-AGGCGGACTATGACTTAGTTGCGTTACACC-3′	5′-AAGTCCTCGGCCACATTGTGAACTTTG-3′	55	Internal control
α-fetoprotein (*AFP*)	5′-CCACTTGTTGCCAACTCAGTGA-3′	5′-TGCAGGAGGGACATATGTTTCA-3′	70	Endodermal marker
GATA binding protein 6 (*GATA6*)	5′-CCTGCGGGCTCTACAGCAAGATGAAC-3′	5′-CGCCCCTGAGGCTGTAGGTTGTGTT-3′	66	Endodermal marker
Microtubule-associated protein 2 (*MAP-2*)	5′-ACTACCAGTTTCACACCCCCTTT-3′	5′-AAGGGTGCAGGAGACACAGATAC-3′	69	Ectodermal marker
NK2 homeobox 5 (*NKX2-5*)	5′-GGGACTTGAATGCGGTTCAG-3′	5′-CTCCACAGTTGGGTTCATCTGTAA-3′	54	Mesodermal marker
Brachyury (*T*)	5′-ACCTTCCATGTGAAGCAGCAA-3′	5′-CTCCACAGTTGGGTTCATCTGTAA-3′	55	Mesodermal marker
Human albumin (*ALB*)	5′-AAATGAAGATCAAAAGCTTAT-3′	5′-TACCGAAGTGGAATAAGAGAGAA-3′	52	Hepatocyte marker

- Filtered pipette tips (1,000-μl; BioPoint Scientific, cat. no. 361-4050)
- Pasteur pipette (5 inch; Iwaki, cat. no. 73-0001)
- Filter (0.22 μm; Millipore, cat. no. SLGV033RS)
- Cryotube (1.8 ml; Thermo Scientific, Nalge Nunc, cat. no. 377267)
- Biofreezing vessel, BICELL (Nihon Freezer)
- Plate rotator
- CO_2 incubator set to 5% CO_2 and 37°C (Thermo Fisher Scientific)
- Tissue culture hood (Showa Science)
- Cell culture centrifuge machine (Kubota, cat. no. Model 5800)
- Microcentrifuge machine (Kubota, cat. no. Model 3700)
- Hemocytometer (OneCell, cat. no. OC-C-S02)
- Inverted microscope equipped with phase-contrast objectives (phase ×4, ×10 and ×20) and charge-coupled device (CCD) system (Nikon)
- Deep freezer set to − 80 °C
- Cryogenic refrigerator (Taylor-Wharton, cat. no. VHC35)
- Microtube mixer (Tomy Digital Biology, cat. no. MT-360)
- Sterile mesh filter (Cell strainer, 100-μm-pore; BD Flacon, cat. no. 352350)
- Fluorescence-activated cell sorter SORP FACSAria II (Becton Dickinson)
- Glass micropipette (Drummond, cat. no. 2-000-050)
- Chemical spatula or micro forceps
- Cryomold (Sakura Finetek, cat. no. TissueTek 4557)
- Cryostat (Leica, cat. no. CM1950)
- Hair dryer
- Coplin jars
- Electrophoresis system, Mupid-2plus (Advance) or similar
- Collagen-coated 35-mm dish (Sumilon, cat. no. MS-0035K)

REAGENT SETUP

L-Glutamine solution, 200 mM Dissolve 2.92 g of L-glutamine in 100 ml of DDW to obtain 200 mM L-glutamine solution. Pass this solution through a 0.22-μm filter, divide it into 1-ml aliquots and store the aliquots at −30 °C for up to 2 months. In the case of M-cluster formation in single-cell suspension culture or in methylcellulose (MC) culture, add a 1/100 volume of 200 mM L-glutamine to the medium just before use. The working concentration of L-glutamine must be 2 mM.

BSA solution, 5% (wt/vol) Dissolve 2.5 g of BSA in 50 ml of D-PBS to obtain a 5% (wt/vol) BSA solution. Pass this solution through a 0.22-μm filter and store it at 4 °C for up to 2 months.

EDTA solution, 100 mM Dissolve 1.86 g of disodium EDTA in 50 ml of D-PBS to prepare a 100 mM EDTA solution. Pass this solution through a 0.22-μm filter and store it at 4 °C for up to 6 months.

BlockAce solution, 100% Dissolve 4 g of BlockAce powder in 100 ml DDW to obtain a 100% BlockAce solution. Pass this solution through a 0.22-μm filter and store it at −30 °C. This solution can be stored for up to 2 months. Please note that the representation '100%' for the BlockAce solution is defined by the manufacturer, and it cannot therefore be presented as wt/vol or vol/vol.

Phosphate buffer solution, 0.4 M Dissolve 35.8 g of disodium hydrogen phosphate 12-H_2O and 15.6 g of sodium dihydrogenphosphate dihydrate in 400 ml of DDW. Titrate the solution with 1 N sodium hydroxide solution to adjust the pH 7.4 and add DDW up to 500 ml to obtain a 0.4 M phosphate buffer (0.4 M PB) solution. Store this solution at room temperature (RT; 18–28 °C). This solution can be stored for up to 2 months.

Fibronectin solution, 1 μg ml^{-1} Dissolve 1 mg of fibronectin in 10 ml of D-PBS. Divide this solution into 50-μl each aliquots and store them at −30 °C for up to 2 months.

bFGF solution, 30 μg ml^{-1} Dissolve 10 μg of bFGF in 333 μl of D-PBS containing 1% (wt/vol) BSA. Divide this solution into 10-μl aliquots and store them at −30 °C for up to 2 months.

EGF solution, 30 μg ml^{-1} Dissolve 60 μg of EGF in 2 ml of D-PBS containing 1% (wt/vol) BSA, divide it into 10-μl aliquots and store the aliquots at −30 °C for up to 2 months.

BDNF solution, 25 μg ml^{-1} Dissolve 10 μg of BDNF in 400 μl of D-PBS containing 1% (wt/vol) BSA. Divide the solution into 10-μl aliquots and store them at −30 °C for up to 2 months.

Dexamethasone solution, 10 μg ml^{-1} Dissolve 1 mg of dexamethasone in 1 ml of ethanol. After dissolving, add 99 ml of α-MEM to the solution. Divide it into 1-ml aliquots and store them at −30 °C. Dexamethasone stock solution can be stored for 2 months.

HGF solution, 100 μg ml^{-1} Dissolve 10 μg of HGF in 100 μl of D-PBS containing 1% (wt/vol) BSA. Divide it into 5-μl aliquots and store them at −30 °C for 2 months.

FGF-4 solution, 25 μg ml^{-1} Dissolve 25 μg of FGF-4 in 1 ml of D-PBS containing 0.1% (wt/vol) BSA. Divide it into 10-μl aliquots and store them at −30 °C for 2 months.

Cell culture medium Mix 50 ml of FBS, 5 ml of 100× Kanamycin sulfate (1× final concentration) and 445 ml of α-MEM to obtain α-MEM containing 10% (vol/vol) FBS. Store this solution at 4 °C for up to 1 month. **! CAUTION** For the maintenance and expansion of cells that are just thawed or are in the first two to three passages after thawing, the specific culture medium recommended by the supplier should be used.

FACS buffer Just before use, mix 5 ml of 5% (wt/vol) BSA solution, 1 ml of 100 mM EDTA solution, 2.5 ml of 100% BlockAce solution and 41.5 ml of D-PBS to obtain 50 ml of FACS buffer.

poly-HEMA solution Pour 38 ml of 99.5% (vol/vol) ethanol and 2 ml of DDW into a 50-ml conical tube and mix them. After mixing, add 1.2 g of poly-HEMA into the conical tube and dissolve it using a plate rotator for 5–6 h at 37 °C. Store this poly-HEMA stock solution at 4 °C for up to 2 months. ▲ **CRITICAL** If poly-HEMA is first placed in the conical tube, followed by the addition of 95% (vol/vol) ethanol, poly-HEMA will coagulate and take extraordinarily long time to dissolve. In addition, poly-HEMA will not dissolve in 100% (vol/vol) ethanol. Thus, 95% (vol/vol) ethanol should

be prepared first in a conical tube and then poly-HEMA should be added. See TROUBLESHOOTING for details.

PFA, 4% (wt/vol)/0.1 M PB solution To prepare the fixative, stir 4 g of PFA into 300 ml of DDW in the water bath set to 80 °C. Titrate with several drops of 2 N sodium hydroxide solution to dissolve the PFA. After dissolving, add 125 ml of 0.4 M PB to the solution and add DDW to make up the total volume to 500 ml. Store this fixative at 4 °C until use. This fixative can be stored for 1 month.

Saline solution Dissolve 9 g of sodium chloride in 100 ml of DDW. Store this solution at 4 °C for up to 6 months.

Blocking solution for immunocytochemistry To make 100 ml of blocking solution for immunocytochemistry, add an appropriate amount of DDW to a mixture containing 20 ml of 100% (vol/vol) BlockAce, 5 g of BSA, 300 µl of Triton X-100, 5 ml of 0.4 M PB and 0.8 g of sodium chloride, and mix well to dissolve them to yield 20% (vol/vol) BlockAce/5% (wt/vol) BSA/0.3% (vol/vol) Triton X-100/0.02 M D-PBS. Store this blocking solution at 4 °C for up to 2 months. Discard this solution if a deposit appears.

Antibody diluent for immunocytochemistry To make 100 ml of antibody diluent for immunocytochemistry, namely, 5% (vol/vol) BlockAce/1% (wt/vol) BSA/0.3% (vol/vol) Triton X-100/0.02 M D-PBS, add an appropriate amount of DDW to the mixture of 5 ml of 100% BlockAce, 1 g of BSA, 300 µl of Triton X-100, 5 ml of 0.4 M PB and 0.8 g of sodium chloride; mix well to dissolve. Store this antibody diluent at 4 °C for up to 2 months. Discard this solution if a deposit appears.

RT-PCR Dissolve the lyophilized powder of each primer in DDW to obtain a 10 mM primer solution. Store the primer solution at –20 °C for up to 1 year.

Gelatin solution Dissolve 0.1 g of gelatin in 100 ml of D-PBS and autoclave the solution to obtain a 0.1% (wt/vol) gelatin solution. Store the solution at 4 °C for up to 2 months.

EQUIPMENT SETUP

Poly-HEMA–coated culture dish Apply an appropriate volume of the poly-HEMA solution (**Table 3**) to each dish or well in the tissue culture hood and make sure to spread the poly-HEMA solution over the entire surface of each dish or well. The lid of the dish should be left fully opened overnight to allow the poly-HEMA solution to completely dry out.
The poly-HEMA–coated dishes can be used for 3 months after coating when stored at RT in the dark. ▲ **CRITICAL** Do not turn on the UV lamp or use the blower while the poly-HEMA solution is drying in the tissue culture hood. These procedures will cause improper coating of the dish. Furthermore, do not try to dry too many dishes at the same time under the same hood because evaporated ethanol will become saturated in the hood and the poly-HEMA solution will not dry out completely.

TABLE 3 | Required amount of poly-HEMA solution for dish coating.

Dish size	Volume of poly-HEMA solution (per dish or per well)
90 mm (diameter)	3.2 ml
60 mm (diameter)	1.3 ml
35 mm (diameter)	500 µl
12 well	200 µl
24 well	100 µl
48 well	70 µl
96 well	25 µl

FACS setup The special-order research products (SORP) BD FACSAria II cell sorter is equipped with four solid-state lasers (laser outputs at 355 nm, 405 nm, 488 nm and 640 nm). For Muse cell isolation by the cell sorter, FITC- and DyLight649-labeled secondary antibodies are used. The signal elicited from FITC is excited by a 488-nm laser and detected by a 530/30-nm band-pass filter passing through a 570-nm long-pass filter, and that from DyLight649 is excited by a 640-nm laser and detected by a 670/30-nm band-pass filter, which rebounds from a mirror with a 690-nm long-pass filter. Thus, the cell sorter applied to the following procedures should be equipped with at least two laser-detector sets that are the same as or similar to those mentioned above. FACSDiva 6.1 software is used for collection, storage and analysis of the flow cytometry data and for sorting the $CD105^+/SSEA-3^+$ cell fraction. The cells immunostained only with the secondary antibodies are used as the negative control.

PROCEDURE

Preparation of adult human dermal fibroblasts or BMSCs ● TIMING 4 h–3 weeks

1| If you are using adult human dermal fibroblasts or BMSCs, follow option A (thawing, culturing, passaging and preparation for FACS analysis). Alternatively, follow option B for preparation of fresh bone marrow–derived mononuclear cells.
▲ **CRITICAL STEP** Fresh bone marrow–derived mononuclear cells are to be used for confirming that Muse cells can be collected from tissues or organs and are not emerging cells under the specific culture condition. Toward this aim, fresh bone marrow–derived mononuclear cells should not be cultured before sorting.

(A) Preparation of adult human dermal fibroblasts ● TIMING 2–3 weeks

(i) *Thawing and culturing.* Before thawing frozen mesenchymal cells, prepare the following solutions in 15-ml conical tubes: 10 ml of α-MEM containing 10% (vol/vol) FBS for thawing frozen mesenchymal cells and 10 ml of the medium recommended by the supplier (or 10 ml of α-MEM containing 10% (vol/vol) FBS) for culturing cells.
! CAUTION If there is no information regarding the culture medium from the cell supplier, α-MEM containing 10% (vol/vol) FBS can be used.

(ii) Warm the cryovial containing purchased frozen cells (see Reagents) in the water bath set to 37 °C to thaw the cells.
▲ **CRITICAL STEP** To avoid contamination by bacteria in the warmed water, take care not to allow the cryovial lid to touch the water level. This is the most crucial step to avoid contamination.

(iii) When the frozen solution is partially thawed, remove the cryovial from the water bath. Wipe the water from the surface of the cryovial and then disinfect the cryovial by spraying it with 70% (vol/vol) ethanol.

(iv) Gently and carefully pipette the partially melted frozen solution using a 1,000-µl pipette in order to melt the remaining frozen solution. Transfer it into the 15-ml conical tube containing 10 ml of α-MEM with 10% (vol/vol) FBS, centrifuge it at 420*g* for 5 min (at ~6 °C), remove the supernatant to leave about 100 µl of the solution and break the cell pellet by gentle and careful tapping.

Box 1 | Making frozen stock for mesenchymal cells ● TIMING 1 h

1. Follow Step 1A(viii–x) of the main PROCEDURE.
2. Remove the supernatant to leave ~100 μl of the solution. Add 900 μl of cryopreservative medium by gentle pipetting, transfer the cryopreservative medium containing the cells into a cryotube, and then close the lid tightly to avoid contamination.
▲ **CRITICAL STEP** Use Cell Banker 1 Plus as the cryopreservative medium. Cryopreservation of cells using culture medium containing 5–10% (vol/vol) DMSO will markedly lower the cell viability.
3. Put cryotubes into the BICELL device and freeze them at −80 °C. On the next day, transfer the cryotubes from the BICELL to a cryogenic refrigerator that contains an appropriate amount of liquid nitrogen for preservation.
▲ **CRITICAL STEP** Use the BICELL device as the chamber for freezing cells because this device enables slow-speed freezing. Rapid freezing will destroy the cells. The combination of Cell Banker 1 Plus and BICELL will give the best result in terms of cell viability.

(v) Gently add the appropriate amount of the medium recommended by the supplier or 10 ml of α-MEM containing 10% (vol/vol) FBS, and then transfer it onto a culture dish of the size recommended by the supplier.
(vi) Incubate the cells overnight in a CO_2 incubator set to 5% CO_2 and 37 °C, and then change the medium. If the dish contains many floating cells, which are considered dead cells, wash the dish with fresh medium twice to remove them. This cell population is P1 (passage 1). Passaging and freezing cells should be performed according to the supplier's instructions.
▲ **CRITICAL STEP** Thawing and culturing (Step 1A(i–vi)) has a substantial effect on the warranty of the product; therefore, the procedures for thawing, growing, passaging and freezing the cells should be performed according to the supplier's instructions until a sufficient amount of frozen stock has been obtained. If the protocol for thawing, growing, passaging and freezing is not provided with the cells, consult the supplier. Alternatively, these procedures may be performed in accordance with the protocols described in this paper at your own risk. After obtaining a sufficient number of cells (P3), proceed to the following steps.
? TROUBLESHOOTING
(vii) When the cells reach 90% confluency, proceed with passaging for expansion (next step). If you wish to freeze cells reaching 90% confluency at any stage after this, follow **Box 1**. If you wish to evaluate and select the best serum match for culturing mesenchymal cells, perform a lot check as described in **Box 2** (recommended if cells are passaged). Estimate the confluency in accordance with **Figure 3**.
▲ **CRITICAL STEP** If the cells reach 100% confluency or greater, cell growth will deteriorate markedly after passaging. Generally, it is best to passage the cells after they reach 90% confluency.

Box 2 | Selecting serum by lot check ● TIMING 2 weeks

1. Collect more than 20 different types of sera that are supplied from different producers and are in different lots.
2. Follow Step 1A(viii–x) of the main PROCEDURE. Prepare a series of 500-μl aliquots of α-MEM containing 10% (vol/vol) sera that are being lot-checked.
3. Remove the supernatant to leave ~100 μl of the solution and break the cell pellet by gentle and careful tapping. Add 900 μl of α-MEM and suspend the cells with the solution by gentle pipetting.
4. Take 8.0 μl of trypan blue using a 10-μl pipette and transfer it into a 1.8-ml microcentrifuge tube. Take 8.0 μl of the above solution containing the cells and transfer it to the same tube. Gently pipette, then transfer 8.0 μl of the mixed solution to a hemocytometer and count the number of viable cells.
5. Suspend the cells in the series of above-prepared 500-μl aliquots of α-MEM/10% (vol/vol) sera at a concentration of 2×10^4 cells per ml, seed them onto individual wells of a 24-well culture dish, and then place the culture plate in a CO_2 incubator set to 5% CO_2 and 37 °C.
6. Change the medium the next day, and continue to change the medium every 2 or 3 d.
7. When the cells reach 90% confluency, which generally comes at 3 d after plating, passage the cells according to the procedure described in Step 1A(viii–x) at a ratio of 1:2 with new medium containing 10% (vol/vol) FBS from the same lot. Allow the cells to grow, and then passage them according to the procedure described in Step 1A(viii–x) again (P2) when they reach 90% confluency. Evaluate the morphology and growth speed of the cells after the third passaging. Select the serum that allows the best growth rate without morphological changes such as flattened, wide cytoplasm, which suggests cell senescence.
▲ **CRITICAL STEP** Because serum used in the cell culture before the lot check may continue to influence cell growth, the morphology and growth speed of the cells should be observed at least up to P3.

(viii) *Passaging*. For passaging cells cultured in a 90-mm tissue culture dish, prepare the following chemical agents individually in 15-ml conical tubes: 10 ml of D-PBS, 2 ml of trypsin-EDTA solution, 1 ml of FBS, 7 ml of α-MEM and 20 ml of α-MEM containing 10% (vol/vol) FBS. Prewarm each solution in the water bath set to 37 °C.

(ix) Remove the culture medium, add 10 ml of D-PBS to the dish for washing and remove the D-PBS again. Apply 2 ml of trypsin-EDTA solution (see Reagents) onto a 90-mm tissue culture dish, tilt the culture dish several times to allow the trypsin-EDTA solution to distribute over the entire surface of the dish and incubate the culture dish in a CO_2 incubator set to 5% CO_2 and 37 °C for 5 min.

(x) Confirm the detachment of the cells from the surface of the culture dish by observation under a phase contrast microscope, and then add 1 ml of FBS to stop the trypsin reaction. Dissociate the cells by gentle pipetting using a 1,000-μl pipette, and transfer the solution containing the dissociated cells into a 15-ml conical tube. To suspend and collect the remaining cells, add 7 ml of new α-MEM to the dish and transfer it to the conical tube to obtain a total of 10 ml of the solution containing the dissociated cells. Centrifuge the tube at 420*g* for 5 min.

? TROUBLESHOOTING

(xi) Remove the supernatant to leave about 100 μl of the solution. Gently add 10 ml of α-MEM containing 10% (vol/vol) FBS; pipette 5 ml of the solution containing the dissociated cells into each of the two individual 90-mm tissue culture dishes using a 10-ml disposable pipette, and then add 5 ml of new α-MEM containing 10% (vol/vol) FBS into each dish. Incubate these two culture dishes in a CO_2 incubator set to 5% CO_2 and 37 °C.

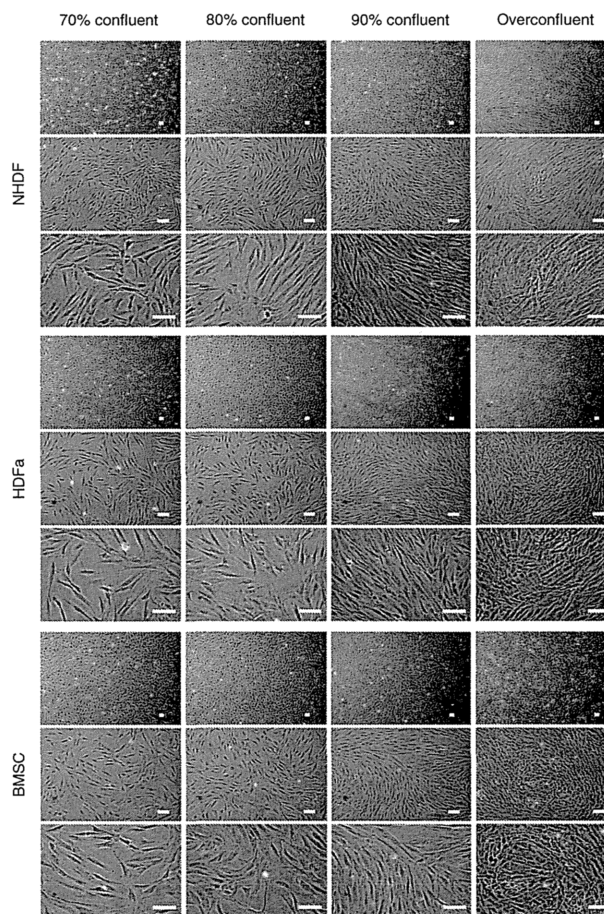

Figure 3 | Cell density and the confluency of mesenchymal cells. Scale bars, 100 μm.

(xii) The next day, change the medium to new α-MEM containing 10% (vol/vol) FBS. If many floating cells are observed before changing the medium, they are considered to be dead cells; in this case, wash the dish with new medium at least twice. In general, the cells reach 90% confluency within 4 d of the latest passage; this is the time for carrying out the next passage. Passaging processes (Step 1A(viii–xii)) take ~1–2 h.

(xiii) *Preparation for FACS*. Allow the cell culture to reach 100% confluency. The cells for FACS isolation should be in the range of P4–P10.

▲ **CRITICAL STEP** Use the cells before P10 for FACS; when cells after P11 are used, the number of SSEA-3⁺ cells or the activity of Muse cells may be much lower than expected.

▲ **CRITICAL STEP** Frozen cells should not be directly applied to Muse cell isolation by FACS. The number of Muse cells in the thawed cell population varies substantially before the first passaging after thawing and will occasionally be too small to obtain a sufficient number of Muse cells. Thus, the cells should be passaged more than once and then applied to FACS.

(xiv) For FACS isolation of Muse cells from cells cultured in a 90-mm dish, prepare the following chemical reagents individually in 15-ml conical tubes: 10 ml of D-PBS, 2 ml of trypsin-EDTA solution, 1 ml of FBS and 7 ml of α-MEM. Prewarm the reagents in the water bath set at 37 °C.

(xv) Remove the culture medium, add 10 ml of D-PBS to the dish for washing and remove D-PBS again. Apply 2 ml of trypsin-EDTA solution (see Reagents) onto a 90-mm culture dish, tilt the dish several times to spread the trypsin-EDTA solution to the entire surface of the dish, and then incubate it in a CO_2 incubator set to 5% CO_2 at 37 °C for 5 min to allow the trypsin reaction to occur.

(xvi) Confirm the detachment of the cells from the surface of the culture dish by observation under a phase-contrast microscope, and then add 1 ml of FBS to stop the trypsin reaction. Dissociate the cells by gentle pipetting with a 1,000-μl pipette, and then transfer the medium containing the dissociated cells into a 15-ml conical tube. Suspend the cells

remaining on the surface of the culture dish in 7 ml of fresh medium, and then transfer them to the 15-ml conical tube to obtain a total of 10 ml of the medium containing the dissociated cells. Centrifuge the tube at 420*g* for 5 min.

(xvii) Remove the medium, add 10 ml of D-PBS to the tube for washing, centrifuge the tube at 420*g* for 5 min, and then remove the D-PBS again. Suspend the cells in 10 ml of FACS buffer, centrifuge the tube at 420*g* for 5 min and remove the supernatant.

(xviii) Suspend the cells in the centrifuge tube in 1 ml of FACS buffer. 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 containing the cells, transfer it into the same microcentrifuge tube, mix the solution, transfer 8.0 μl of this solution to a hemocytometer and count the cell number. FACS processes (Step 1A(xiii–xviii)) take ~1 d.

(B) Preparation of fresh bone marrow–derived mononuclear cells ● TIMING **4 h**

(i) Sterilize 5-inch-long Pasteur pipettes by autoclaving.

(ii) If fresh bone marrow provided by ALLCELLS is used, add 4 ml of D-PBS into 16 ml of bone marrow aspirate provided by ALLCELLS (consisting of 10 ml of bone marrow aspirate, 6 ml of D-PBS and 800 units of heparin). Mix it by gentle pipetting to obtain 20 ml of the solution containing the bone marrow aspirate, D-PBS and heparin. If bone marrow aspirates provided from other companies or prepared in-house are used, prepare 20 ml of the mixture of bone marrow aspirates, D-PBS and anticoagulants such as EDTA, heparin or acid citrate dextrose, in which the volume of D-PBS is equal to or greater than that of the bone marrow aspirates.
▲ **CRITICAL STEP** If bone marrow aspirates provided from other companies or prepared in-house are used, do not forget to mix the preparation with an anticoagulant. The concentration of anticoagulant used should be optimized by your own hand.

(iii) Centrifuge the Lymphoprep tubes at 400*g* for 1 min (at ~6 °C, in this and other centrifugation steps unless otherwise indicated) to displace the liquid inside to the bottom of the tube underneath the partitioning membrane; a Lymphoprep tube allows the separation of mononuclear cells from 25 to 35 ml of the mixture of the bone marrow aspirate and D-PBS. Gently apply the mixture of the bone marrow aspirate and D-PBS onto the partitioning membrane in the Lymphoprep tube and centrifuge at 800*g* for 30 min in a swing-out rotor. After centrifugation, a distinct band of mononuclear cells will emerge at the sample/medium interface (refer to the schematic figure in the manufacturer's brochure: http://www.axis-shield-density-gradient-media.com/leaflet%20Lymphoprep%20Tube.pdf).
▲ **CRITICAL STEP** Cell viability is the most important factor to be considered in isolation of mononuclear cells from fresh bone marrow. Therefore, it is better to isolate mononuclear cells as soon as possible after the aspiration of bone marrow.
▲ **CRITICAL STEP** When centrifuging the Lymphoprep tube, the rate of deceleration in the cell culture centrifuge machine should be set to the minimum value. If it is not properly set, the distinct band of mononuclear cells will not appear.

(iv) Collect the specific band containing mononuclear cells by aspiration using a sterilized 5-inch-long Pasteur pipette. Transfer it into a new 50-ml conical tube and add 20 ml of α-MEM containing 10% (vol/vol) FBS. Centrifuge it at 420*g* for 5 min, discard the supernatant, add 10 ml of D-PBS to the tube for washing, centrifuge the tube at 420*g* for 5 min, and then remove the D-PBS again.

(v) Perform hemolysis using red blood cell lysis buffer according to the manufacturer's instructions. Briefly, pour 1 ml of red blood cell lysis buffer and 9 ml of DDW into a new 15-ml conical tube, mix them by pipetting and pour the solution into the 50-ml tube containing mononuclear cells to suspend them. Close the lid of the tube, mix the solution well by inverting the tube, and then incubate the tube for 10 min at RT. After incubation, centrifuge the cell suspension at 300*g* for 10 min, discard the supernatant, suspend the cells in 20 ml of α-MEM supplemented with 10% (vol/vol) FBS for washing, centrifuge the tube again at 300*g* for 10 min, and then discard the supernatant again.
▲ **CRITICAL STEP** In the best case, the cell pellet will be whitish, not reddish, after centrifugation. Occasionally, two layers will be formed: a white and muddy upper layer and a red lower layer, suggesting insufficient hemolysis. This usually depends on the time from aspiration to treatment. Nevertheless, even if the hemolysis seems insufficient, multiple treatments of mononuclear cells with red blood cell lysis buffer will substantially reduce cell viability; therefore, the hemolysis treatment should be performed only once.

(vi) Suspend the cells in 1 ml of FACS buffer, transfer the suspension to a new 1.8-ml microcentrifuge tube, centrifuge it at 420*g* for 5 min, discard the supernatant and add 1 ml of 10% (vol/vol) heat-inactivated human serum containing FACS buffer at 4 °C for 20 min to block the nonspecific binding of antibodies to Fc receptors that are expressed on the surface of the cells in the mononuclear cell fraction. After incubation, centrifuge the sample at 420*g* for 5 min, and discard the supernatant.

(vii) Suspend the cells in the tube in 1 ml of FACS buffer. 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 containing cells, transfer it into the same microcentrifuge tube, mix the solution, and then take 8.0 μl of this solution to the hemocytometer and count the number of live cells.

Isolation of Muse cells by FACS ● TIMING **4–6 h**

2| Centrifuge the tube containing the cells for Muse cell isolation at 420*g* for 5 min, discard the supernatant and suspend the cells with FACS buffer at $<1.0 \times 10^6$ cells per 100 μl. Divide it into the appropriate number of samples such as: no staining (tube a), only secondary antibody staining (tube b) and actual staining for SSEA-3 (tube c; staining with both primary and secondary antibodies). Use 1.8-ml microcentrifuge tubes for these aliquots.

! CAUTION For large-scale yields of Muse cells for experiments such as transplantation studies or for iPS cell generation[39,40], expand the mesenchymal cell population as much as possible and isolate Muse cells by FACS. This is the simplest and most practical method for obtaining a large number of Muse cells (rather than obtaining Muse cells by cycle culture).

▲ **CRITICAL STEP** Inordinately high cell concentrations ($>1.0 \times 10^6$ cells per 100 μl) lead to insufficient cell staining, in turn resulting in a reduction in the number of SSEA-3$^+$ cells. Concentrations of not more than 1.0×10^6 cells per 100 μl are strongly recommended.

3| Add anti–SSEA-3 antibody to the microcentrifuge tube (tube c in Step 2). When mononuclear cells separated from fresh bone marrow are applied to FACS separation of Muse cells, add anti-CD105 antibody in addition to anti–SSEA-3. The volume of both the anti–SSEA-3 and anti-CD105 antibodies should be 2 μl for 100 μl of FACS buffer (1:50) regardless of the cell concentration. Incubate the cell suspension on ice for 1 h with mixing every 10 min by a microtube mixer set to the maximum mixing speed for 2 s or by gentle pipetting with a 1,000-μl pipette every 10 min.

▲ **CRITICAL STEP** Use the anti–SSEA-3 antibody supplied by Millipore or the number of SSEA-3$^+$ cells will be improperly estimated.

▲ **CRITICAL STEP** Because mononuclear cells separated from fresh bone marrow contain cells other than mesenchymal cell populations, anti-CD105 antibody should be added for discriminating these cells. If cultured mesenchymal cells are used for isolating Muse cells by FACS, anti-CD105 antibody is not required.

4| Centrifuge the tube at 420*g* for 5 min at 37 °C, discard the supernatant and suspend the cells with 1 ml of the FACS buffer for washing. Repeat this step three times. During the incubation, turn on and set up the FACS machine.

▲ **CRITICAL STEP** When discarding the supernatant and suspending the cells in the FACS buffer, leave 100 μl of the supernatant and use a microtube mixer set to the maximum mixing speed for 2 s for suspending. After this, add 1 ml of the FACS buffer.

▲ **CRITICAL STEP** Use a 100-μm nozzle for FACS analysis and sorting.

5| Centrifuge the tube at 420*g* for 5 min at 37 °C, discard the supernatant and add the FACS buffer to a final cell concentration of less than 1.0×10^6 cells per 100 μl. Add anti-rat IgM antibody conjugated to FITC to the microcentrifuge tubes (tubes b and c in Step 2) containing the cells. When mononuclear cells separated from fresh bone marrow are applied to FACS, also add anti-mouse IgG antibody conjugated to DyLight649. The volume of the secondary antibodies (FITC-conjugated anti-rat IgM and DyLight649-conjugated anti-mouse IgG antibody) should be 1.0 μl and 0.5 μl for 100 μl (1:100 and 1:200, respectively) of the FACS buffer regardless of cell concentration. Incubate the cell suspension on ice for 1 h with mixing every 10 min by a microtube mixer set to the maximum mixing speed for 2 s or by gentle pipetting with a 1,000-μl pipette.

6| Centrifuge the tubes at 420*g* for 5 min at 37 °C, discard the supernatant and suspend the cells in 1 ml of the FACS buffer for washing. Repeat this step three times.

7| Pass the cell suspensions through a cell strainer (100-μm pore) to eliminate cell aggregates. Place the microcentrifuge tubes containing cells as the sample and a new 1.8-ml microcentrifuge tube for collection of the sorted cells in the SORP FACSAria II.

8| Determine the SSEA-3$^+$ fraction using the control samples (tubes a and b) (**Fig. 4a**) and then sort the SSEA-3$^+$ cells (tube c) using the SORP FACSAria II (**Fig. 4b–e**). When mononuclear cells separated from fresh bone marrow are applied to FACS separation of Muse cells, the setting for CD105 is also determined with no-staining and only secondary-antibody-only staining samples. When performing double staining, sort the cells that are double positive for SSEA-3 and CD105 as the FITC-positive and DyLight649-positive fractions, respectively, such that there should be no overlap between the excited fluorescence of each fluorescent dye; therefore, it is not necessary to perform compensation for FACS sorting.

▲ **CRITICAL STEP** Set the area-scaling factor; use forward scatter (FSC)-H/FSC-W and side scatter (SSC)-H/SSC-W instead of excluding large-sized cell structures with the FCS parameter. This will help to avoid contamination of doublets or triplets. Because the sizes of mesenchymal cells in primary culture or in fresh bone marrow mononuclear cells vary considerably, it is not possible to detect doublets or triplets by using only the FSC parameter.

▲ **CRITICAL STEP** The yield of Muse cells by FACS sorting is highly dependent on the viability of the cells. To mix the cells during the process of immunostaining for FACS, we highly recommend using a microtube mixer to mix the solution containing the cells instead of performing gentle pipetting. The gentleness of pipetting is highly dependent on the person who performs it, but mixing by a microtube mixer is reproducible and gives consistent results. Careless mixing of cells will

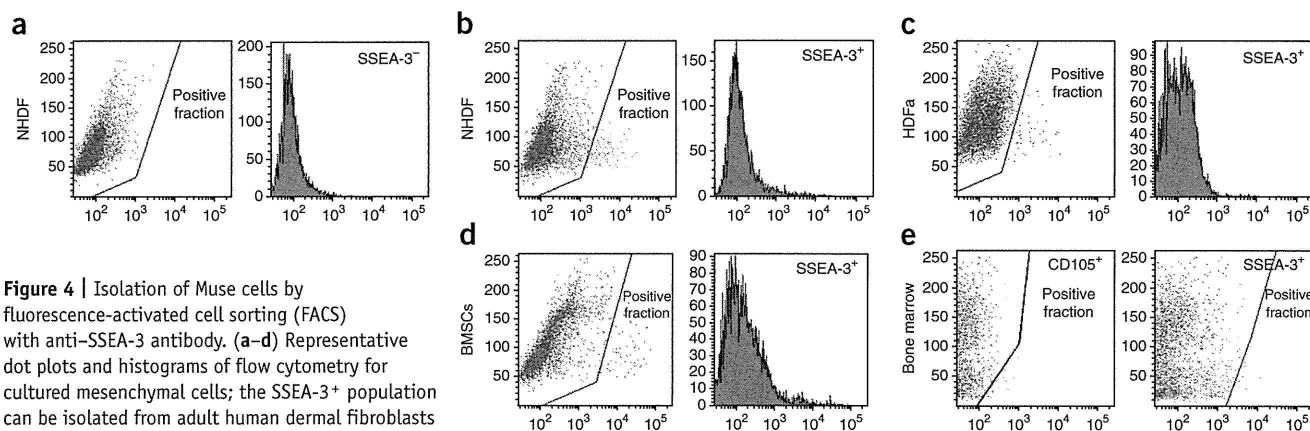

Figure 4 | Isolation of Muse cells by fluorescence-activated cell sorting (FACS) with anti–SSEA-3 antibody. (**a–d**) Representative dot plots and histograms of flow cytometry for cultured mesenchymal cells; the SSEA-3$^+$ population can be isolated from adult human dermal fibroblasts (**a–c**) and adult 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

(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.

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

(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)

TABLE 5 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
	No differentiation or too-low efficiency in differentiation	Improper cell density	Perform induction of differentiation in an appropriate cell density according to the protocol
20A(iv), 20C(ii)	The signal for the specific cell type cannot be detected in cultured cells on a gelatin-coated coverslip	Prolonged culturing	Do not culture cells on a gelatin-coated coverslip until they reach 100% confluency or specific cell types will be lost

● TIMING

Step 1, preparation of adult human dermal fibroblasts or bone marrow stromal cells: 4 h–3 weeks
Step 1A, preparation of adult human dermal fibroblasts: 4 h–3 weeks
Step 1A(i–vii), thawing and culturing: 2–3 weeks
Step 1A(viii–xii), passaging: 1–2 h
Step 1A(xiii–xviii), preparation for FACS: 1 d
Box 1, making frozen stock for mesenchymal cells: 1 h
Box 2, selecting serum by lot check: 2 weeks
Step 1B, preparation of fresh bone marrow–derived mononuclear cells: 4 h
Steps 2–9, isolation of Muse cells by FACS: 4–6 h
Step 10, M-cluster formation in suspension culture: 7–10 d
Step 10A, single-cell suspension culture: 7–10 d
Step 10B, MC culture: 7–10 d
Steps 11–17, adherent culture: 7–10 d
Step 18, evaluation of Muse cells in M-clusters: 1.5 h–2 d
Step 18A, detection of ALP activity: 1.5 h
Step 18B, immunocytochemistry against pluripotency markers: 2 d
Step 19, evaluation of the differentiation capacity of Muse cells: 1–3 weeks
Step 19A, spontaneous differentiation on a gelatin-coated coverslip: 1–2 weeks
Step 19B, dissociation of M-clusters for induced differentiation of Muse cells into mesodermal-, endodermal- and ectodermal-lineage cells: 1 h
Step 19C, induced differentiation of Muse cells into mesodermal-, ectodermal- and endodermal-lineage cells: 2–3 weeks
Step 19C(ii–vi), osteocyte induction: 2–3 weeks
Step 19C(vii–ix), adipocyte induction: 2–3 weeks
Step 19C(x–xi), hepatocyte induction: 2 weeks
Step 19C(xii–xvi), neural induction: 3 weeks
Step 20, evaluation of cell differentiation: 5 h–2 d
Step 20A, immunocytochemical analysis of the differentiated cells: 2 d
Step 20B, immunocytochemical analysis of the sphere cells: 2 d
Step 20C, RT-PCR: 5 h

ANTICIPATED RESULTS

In the case of isolation of Muse cells from cultured mesenchymal cell populations that can be purchased commercially, adult human BMSCs and dermal fibroblasts should be cultured in the appropriate culture conditions described in the PROCEDURE. Healthy MSCs have long, thin cell bodies with a large nucleus, usually referred to as 'fibroblast-like morphology', and MSCs cultured in a 90-mm tissue culture dish generate 1.0×10^6 BMSCs, 2.0×10^6 NHDFs and 2.1×10^6 HDFas at 100% confluency (**Fig. 3**). In contrast, mononuclear cells derived from fresh bone marrow are another cell source for Muse cells. According to the brochure accompanying the commercial bone marrow preparation, the number of mononuclear cells elicited from 10 ml of fresh bone marrow is calculated as $0.2–1.7 \times 10^8$ cells, but it actually depends on the freshness of the bone marrow aspirates. For example, when we purchased bone marrow from ALLCELLS, which is imported from the United States to Japan and typically takes 5–7 d to arrive, the number of mononuclear cells elicited from the bone marrow aspirate was as low as about $2.0–5.0 \times 10^6$ cells, and sometimes no cells could be collected. We strongly recommend isolating mononuclear cells as soon as possible after aspiration of the bone marrow.