a

Isolation and cultivation of Muse cells from mesenchymal cells by FACS

Step 1, preparation of mesenchymal cells (choose option A or B)

Option A, culture of mesenchymal cell populations (BMSCs, dermal fibroblasts)

Option B, preparation of fresh bone marrow–derived mononuclear cells

Steps 2–9, isolation of Muse cells by FACS

Step 10, M-cluster formation in suspension culture (choose option A or B)

Option A, single-cell suspension culture

Option B, MC culture

Steps 11–17, adherent culture

b

Evaluation of the self-renewal property of Muse cells

Step 1, preparation of mesenchymal cells (choose option A or B)

Option A, culture of mesenchymal cell populations (BMSCs, dermal fibroblasts)

Option B, preparation of fresh bone marrow–derived mononuclear cells

Steps 2–9, isolation of Muse cells by FACS

Step 10A, M-cluster formation in suspension culture

Step 18, evaluation of Muse cells in M-clusters

Step 19A, spontaneous differentiation on a gelatin-coated coverslip

Step 20, detection of cell differentiation marker

Steps 11–17, adherent culture

Steps 2–9, isolation of Muse cells by FACS

c

Evaluation of the triploblastic differentiation capacity of FACS-isolated Muse cells

Step 1, preparation of mesenchymal cells (choose option A or B)

Option A, culture of mesenchymal cell populations (BMSCs, dermal fibroblasts)

Option B, preparation of fresh bone marrow–derived mononuclear cells

Steps 2–9, isolation of Muse cells by FACS

Step 19, differentiation of FACS-isolated Muse cells (choose option A or C)

Option A, spontaneous differentiation on a gelatin-coated coverslip

Option C, induced differentiation into mesodermal, endodermal and ectodermal lineages

Step 20, detection of cell differentiation markers

d

Evaluation of the triploblastic differentiation capacity of Muse cells in M-clusters

Step 1, preparation of mesenchymal cells (choose option A or B)

Option A, culture of mesenchymal cell populations (BMSCs, dermal fibroblasts)

Option B, preparation of fresh bone marrow–derived mononuclear cells

Steps 2–9, isolation of Muse cells by FACS

Step 10B, MC culture

Step 19, differentiation of Muse cells derived from M-clusters (choose option A or B plus C)

Option A, spontaneous differentiation on a gelatin-coated coverslip

Option B plus option C, induced differentiation

Step 20, detection of cell differentiation markers

Figure 1 | Schematic overview of isolation, cultivation and evaluation of Muse cells.

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Their telomerase activity is as low as that detected in adult dermal fibroblasts, and they do not form teratomas when transplanted into the testes of immunodeficient mice, as is often observed after transplantation of ES cells or iPS cells. *In vivo,* Muse cells can integrate into damaged tissues and spontaneously differentiate into tissue-specific marker–expressing cells according to the micro-environment when transplanted into animal models of fulminant hepatitis, skeletal muscle injury and skin injury[39]. Therefore, Muse cells are suggested to be the 'repairing cells' that have been postulated to exist among MSCs[39]. Another remarkable advantage of Muse cells is that they can be an efficient and practical cell source for iPS cell generation[40]. Adult human dermal fibroblast–derived Muse cells generated iPS cells with 30-fold higher efficiency than naive fibroblasts did, whereas cells other than Muse cells failed to form iPS cells, suggesting that Muse cells are virtually the original cell source for iPS cells among human dermal fibroblasts[40]. The unique capacities of Muse cells, including triploblastic differentiation, self-renewal, tissue repair without tumorigenic activity and high iPS cell–generation efficiency have great benefits not only in regenerative medicine but also in basic research into, for example, the full characterization of MSCs and the mechanisms of iPS cell generation.

Adult human mesenchymal tissues such as the bone marrow and dermis are known to contain several kinds of stem or progenitor cells such as multipotent adult progenitor cells, very small embryonic-like stem cells, marrow-isolated adult multilineage inducible cells, skin-derived precursors and CD146$^+$ osteoprogenitors[18,41–47], some of which are known to express pluripotency markers and are reported to have triploblastic differentiation ability similar to that of Muse cells[18,41–45]. However, the triploblastic differentiation ability of these stem cells has not been demonstrated at a single-cell level, as has been shown in Muse cells[40], so that pluripotency in the above-mentioned cell types has been, in the strict sense, an open question. The expression pattern of cell surface antigens in these stem cells is quite different from that in Muse cells, and, in particular, none of these stem cells express SSEA-3, by which Muse cells can be isolated directly from the adult mesenchymal tissue such as fresh bone marrow. Besides, when transplanted into the injured or degenerative tissues, Muse cells show high tissue-repairing activity, by which 80% or more Muse cells can differentiate into tissue-specific cell types, and they can replenish lost cells in damaged tissues[39]. A similarly high tissue-repairing activity has not been reported in the above-mentioned stem or progenitor cells. Thus, Muse cells are considered a distinct cell population from previously reported stem or progenitor cells among mesenchymal cell populations. Although some of the properties of Muse cells, namely self-renewal, triploblastic differentiation *in vitro* and tissue-repairing effect *in vivo,* have been clarified[39,40], future studies are required to elucidate the specific behavior of endogenous Muse cells, including the self-renewal property that has been demonstrated in a particular cell population of BMSCs[46,47], as well as their broad-ranged differentiation, the biological significance of the expression of pluripotency markers and the correlation between Muse cells and other tissue stem or progenitor cells.

Experimental design

We present here the protocol for isolation, cultivation and evaluation of Muse cells. A summary of our protocol is shown in **Figure 1**. We first describe the methods for preparation of mesenchymal cells (Step 1). In this section, the detailed protocol for culturing BMSCs and dermal fibroblasts is described (Step 1A), because the viability and isolation efficiency of Muse cells are highly dependent on the basic culture method of these mesenchymal cells. As Muse cells can be isolated from mononuclear cells from fresh bone marrow, the protocol for preparing

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.

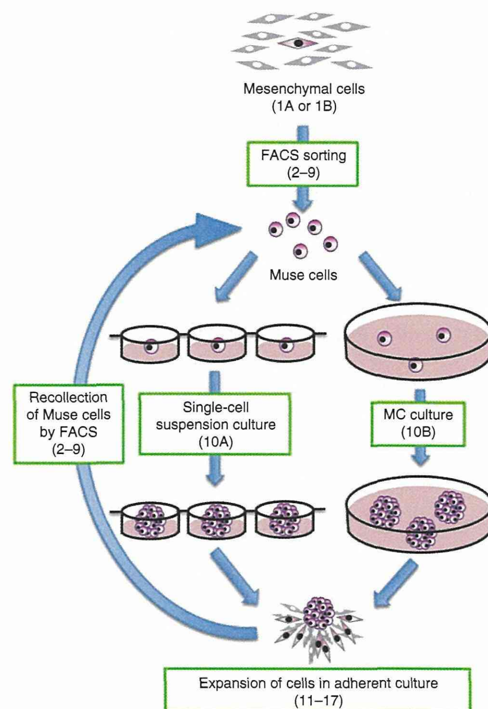

Figure 2 | Schematic diagram of the self-renewal of Muse cells. Numbers in parentheses correspond to Step numbers in the PROCEDURE.

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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 anti-body; 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
Immunocy-tochemistry	Primary	Nanog	Rabbit	Polyclonal	Chemicon	AB5731	1:500		Pluripotency marker
Immunocy-tochemistry	Primary	Oct3/4	Rabbit	Polyclonal	Gift from H. Hamada		1:800		Pluripotency marker
Immunocy-tochemistry	Primary	Sox2	Rabbit	Polyclonal	Abcam	ab59776	1:1,000		Pluripotency marker
Immunocy-tochemistry	Primary	PAR4	Mouse	Monoclonal, A-10, IgG2a	Santa Cruz Biotechnology	sc-1666	1:100		Pluripotency marker
Immunocy-tochemistry	Primary	Neuro-filament	Rabbit	Polyclonal	Chemicon	AB1987	1:200		Neuronal (ectodermal) marker
Immunocy-tochemistry	Primary	Smooth muscle actin	Mouse	Monoclonal, 1A4	LabVision	MS-113-P0	1:100		Smooth muscle cell (mesodermal) marker
Immunocy-tochemistry	Primary	α-fetoprotein	Rabbit	Polyclonal	DAKO	N1501	1:100		Hepatocyte (endodermal) marker
Immunocy-tochemistry	Primary	Cytokeratin-7	Mouse	Monoclonal, RCK105, IgG1	Chemicon	MAB3226	1:100		Hepatic (endodermal) marker
Immunocy-tochemistry	Primary	Desmin	Mouse	Monoclonal, RD301, IgG2b	BD Biosciences	550626	1:100		Muscle cell (mesodermal) marker
Immunocy-tochemistry	Primary	Osteocalcin	Mouse	Monoclonal, 190125, IgG1	R&D Systems	MAB1419	1:10		Osteocyte (mesoder-mal) marker
Immunocy-tochemistry	Primary	Nestin	Mouse	Monoclonal, Rat-401, IgG1	Millipore	MAB353	1:100		Neural progenitor (ectodermal) marker
Immunocy-tochemistry	Primary	Musashi	Rabbit	Polyclonal	Millipore	AB5977	1:200		Neural progenitor (ectodermal) marker

(continued)

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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 Immuno-Research Laboratories	112-095-075	1:100	FITC	Detection of the SSEA-3 primary antibody

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- 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

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- 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 $CD105^{+}/SSEA\text{-}3^{+}$ cell fraction. The cells immunostained only with the secondary antibodies are used as the negative control.

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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.

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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.

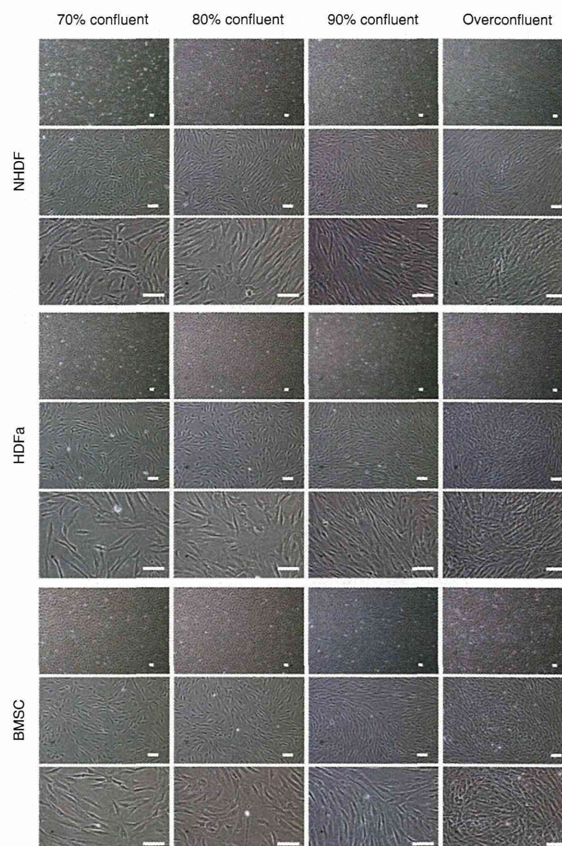

Figure 3 | Cell density and the confluency of mesenchymal cells. Scale bars, 100 μm.

(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

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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.

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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 × 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 × 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.

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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