Supporting Information

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SI Materials and Methods

All animal experiments were approved by the Animal Care and Experimentation Committee of Tohoku University Graduate School of Medicine and Kyoto University Graduate School of Medicine. The human ES (hES) cell line Kyoto hESC-1 (KhES-1) was obtained from the Institute for Frontier Medical Science, Kyoto University, with approval for hES cell use granted by the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The Review Board of the Graduate School of Medicine, Kyoto University, approved this research. The entire study was conducted in accordance with the Declaration of Helsinki. KhES-1 was maintained, as described previously (1).

Method 1: Culture Cells. Two strains of human skin fibroblasts and four strains of H-MSCs were used in this study. H-fibroblast strains were H-fibroblast-1 [normal H-fibroblast cells (Lonza)] and H-fibroblast-2 [adult human dermal fibroblast cells (ScienCell)]. H-MSC strains, H-MSC-1, -2 and -3, were obtained from Lonza, and H-MSC-4 was obtained from ALLCELLS. Cells were maintained at 37 °C in α-MEM containing 10% (vol/vol) FBS and 0.1 mg/mL kanamycin. Three hBM aspirates from healthy donors were purchased from ALLCELLS, and mononucleated cells were collected using Lymphoprep Tubes (Axis-Shield PoC AS).

Cells cultured directly after their shipment were considered to be the first culture. When cells reached 95% confluence, they were expanded at a ratio of 1:2 (second subculture). In this study, cells from the fourth to 10th subcultures were used. Mouse ES cells (TT2 cells) and KhES-1 were maintained on mouse embryonic fibroblast (MEF) cells established from 12.5-day embryos of C57BL/6 mice, as described previously (2).

Method 2: Stress Conditions for Mesenchymal Cells. To estimate stress conditions that may enrich putative multipotent stem cells, we explored poor nutrition, low serum, low O_2, repetitive trypsin treatments, and LTT. The six conditions we tested were (*i*) culture in nonserum-containing medium (STEMPRO MSC SFM; Invitrogen) for 2 days, (*ii*) culture in HBSS buffer (Invitrogen) for 2 days, (*iii*) culture in 10% (vol/vol) FBS in α-MEM combined with low O_2 (1%) for 2 days, (*iv*) three consecutive 1-hr incubations with trypsin (0.25% trypsin-HBSS; Invitrogen), (*v*) LTT for 8 hr, and (*vi*) LTT for 16 hr. For negative controls, we used a mixture of human peripheral mononuclear cells from four healthy volunteers.

For conditions *iv*, *v*, and *vi*, $\approx 1–5 \times 10^5$ cells were suspended in 5 mL of trypsin solution, transferred to a 6-cm diameter dish, and incubated at 37 °C. When cells to be processed exceeded 500,000 cells, they were divided into several groups of less than 500,000 cells and then subjected to trypsin incubation, as discussed previously. After the described treatments, cells from conditions *i* through *iii* were collected by means of a 5-min trypsin treatment and cells from conditions *iv* through *vi* were transferred directly to tubes.

Severe stress conditions produce large numbers of dead cells. Toxic effects exerted by these dead cells can significantly affect the survival and growth of live cells, interfering with the analysis. It would therefore not have been ideal to culture the cells on MC (described below) directly after subjecting them to stress conditions. We therefore tested whether vortexing could be used to break down dead cells. We evaluated and adjusted parameters, such as the volume of medium, the type of tube, and the strength and duration of the vortexing. We found that the best way to eliminate dead cells was to transfer a maximum of 500,000 cells in 5 mL of medium into a 15-mL Falcon tube, continuously vortex the tube for 3 min by MS1 Minishaker (IKA Works, Inc.) at 1,800–2,200 rpm/min, centrifuge at 740 × *g* for 15 min, and finally remove the supernatant containing the dead cells.

Method 3: MC Culture. Culture dishes were first coated with polyHEMA [poly(2-hydroxyethyl methacrylate), P3932; Sigma] to avoid attachment of cells to the bottom of the dish. In brief, 600 mg of polyHEMA was dissolved in 40 mL of 95% ethyl alcohol by shaking at 37 °C and added to the dish (e.g., 40 μL per well for 96-well plates and 200 μL per well for 12-well plates), and the dish was air-dried in the clean bench overnight.

MC (MethoCult H4100; StemCell Technologies) was diluted in 20% (vol/vol) FBS plus α-MEM to a final concentration of 0.9%. The cell concentration in the semisolid MC medium was adjusted to be 8×10^3 cells per milliliter. Cells and MC were mixed thoroughly by gentle pipetting, and the mixture was transferred to a polyHEMA-coated dish. At this concentration, the cell-to-cell distance was sufficiently large to minimize cell aggregation. To prevent drying, a volume equal to 1/10th of the initial MC culture of 10% (vol/vol) FBS in α-MEM was gently added to the dish every 3 days.

M-clusters were counted on day 7. PBS (0.01 M) was added to the culture medium, the cells were centrifuged at 740 × *g* for 20 min, and the supernatant was discarded. This procedure was repeated three times to wash the cells. The cell pellet was finally suspended in ∼10 μL of 0.01 M PBS containing trypan blue and applied to a glass slide, and the entire area was automatically imaged using phase-contrast microscopy (PALM MicroBeam; PALM Microlaser Technologies). Only multicellular clusters larger than 25 μm that were negative for trypan blue and had an appearance similar to hES cell clusters were counted as M-clusters, as described in the main text. The frequency of M-cluster formation was calculated as the number of M-clusters divided by the number of all live cells (all the trypan blue-negative cells). Because it was not possible to determine the precise number of cells in each aggregate, each M-cluster was counted as one cell, irrespective of its size.

For making cell clusters from hES cells, the hES cells were carefully isolated from feeder cells so as not to include feeder cells, dissociated into small clusters, transferred to MC culture as described above, and imaged by phase-contrast microscopy at day 3. ALP staining and immunocytochemistry were performed at day 4.

Method 4: Single-Cell Suspension Culture. A 96-well dish was coated with polyHEMA, as described above. Following a limiting dilution of cells with 10% (vol/vol) FBS in α-MEM, each single cell was picked up by microglass pipette (Fig. S2) and individually plated into each well. After plating, the actual number of cells deposited in each well was confirmed by visual inspection using a phase-contrast microscope. Empty wells or wells with more than one cell were marked and excluded from the analysis. To evaluate the consistency of single cells, we stained cells with Hoechst (Sigma) 33342 before transfer to 96 wells for single-cell suspension culture and confirmed that only single cells were transferred and the cells rarely contained more than two nuclei. The calculation of M-cluster formation was performed on days 7–10. The frequency of M-cluster formation was calculated from three experiments for each strain, with a minimum of 250 wells per experiment.

Method 5: ALP Staining. hES cells, naive cells, and M-clusters (day 7) from both H-fibroblasts and H-MSCs were washed several times with a sufficient volume of saline. Staining was performed

using a Leukocyte Alkaline Phosphatase kit (Sigma) according to the manufacturer's instructions.

Method 6: In Vitro Differentiation of M-Clusters. After 7–10 days of MC culture or single-cell suspension culture after limiting dilution, single M-clusters from both H-fibroblasts and H-MSCs were picked up with a glass micropipette and transferred onto a gelatin-coated culture dish or cover glass. After another 7 days of incubation, M-clusters were subjected to immunocytochemistry and RT-PCR analyses.

Method 7: Immunocytochemistry. Cells were fixed with 4% (vol/vol) paraformaldehyde in 0.01 M PBS. MEC populations and M-clusters from both H-fibroblasts and H-MSCs were collected by centrifugation and embedded in OCT compound, and ~8-μm-thick cryosections were cut. In vitro-differentiated M-clusters on gelatin-coated cover glasses were fixed with 4% (vol/vol) paraformaldehyde in 0.01 M PBS before immunocytochemistry.

The following primary antibodies were used for immunocytochemistry: Nanog (1:500; Chemicon), Oct3/4 [1:800; kindly provided by H. Hamada (3), Osaka University, Osaka, Japan], Sox2 (1:1,000; Chemicon), PAR4 (1:100; Santa Cruz), SSEA-3 [1:20; Developmental Studies Hybridoma Bank (DSHB), University of Iowa], SMA (1:100; Lab Vision), neurofilament-M (1:200; Chemicon), α-fetoprotein (1:100; DAKO), desmin (1:100; BD Biosciences), and cytokeratin 7 (1:100; Chemicon). These primary antibodies were detected with Alexa-488- or Alexa-568-conjugated anti-rabbit IgG, anti-mouse IgG, or anti-mouse IgM antibody (Molecular Probes).

Samples were incubated with block solution containing 5% (vol/vol) normal goat serum (Vector), 0.1% Triton X-100 (Sigma), and 0.3% BSA (Sigma) in 0.01 M PBS at room temperature for 30 min. Samples were then incubated with primary antibodies in blocking solution overnight at 4 °C. After three washes with 0.01 M PBS containing 0.05% Triton X-100, samples were incubated with secondary antibodies for 2 hr at room temperature, followed by counterstaining with DAPI (for nuclear staining, 1:1,000; Sigma) in PBS containing 0.1% Triton X-100. All images were taken with a confocal laser scanning microscope (CS-1; Nikon) using the same laser intensity and detection sensitivity.

Immunocytochemistry of Oct3/4, Nanog, Sox2, PAR4, and SSEA-3 in M-clusters was repeated at least 30 times with different samples for each marker. The evaluation of in vitro differentiation of M-cluster-derived expanded cells into endodermal, ectodermal, and mesodermal cells by immunocytochemistry (neurofilament-M, SMA, and α-fetoprotein) was repeated 7 times with different samples for each marker. The expression of these markers was reproduced in every M-cluster.

Method 8: Determination of Karyotypes. Karyotypes of cells expanded from M-clusters (first and third generations) from both H-fibroblasts and H-MSCs were identified by quinacrine-Hoechst staining at the International Council for Laboratory Animal Science (ICLAS) Monitoring Center (Kawasaki, Japan).

Method 9: Injection of Cells into the Testes of Immunodeficient Mice. MEC populations and M-clusters from both H-fibroblasts and H-MSCs were used. MEC populations were prepared by adding serum to the cells after LTT to neutralize trypsin, followed by three washes with 0.01 M PBS. M-clusters were collected from MC cultures and also washed three times with PBS. Cells (1×10^5) were suspended in PBS and injected using glass microtubes into the testes of NOG mice [NOD/Shi-SCID (4), IL-2RγKO Jic, 8 weeks old; ICLAS Monitoring Center). The average volume of the cells in M-clusters was measured using the 3D-graphic analysis software provided with the laser confocal microscope (50 clusters were measured, and the total volumes of the clusters were divided by the number of nuclei), which resulted in 1.5×10^5 cells per 1-μL volume of collected M-cluster pellet. Each testis of a NOG mouse was then injected with a volume corresponding to 1×10^5 cells, and the mice were killed for analysis 6 months after the injection.

As a control, 1×10^6 mouse ES cells (for a positive control, $n = 3$) and mitomycin C-treated MEF cells (for a negative control, $n = 3$) were injected into SCID mice testes, and the mice were killed 8 weeks after the injection.

Method 10: Growth Rate of M-Clusters. After LTT, each single cell of the MEC population from both H-fibroblasts and H-MSCs was transferred onto polyHEMA-coated 96-well plates and cultured for single-cell suspension. Two to 14 days after plating, the cell number in each well in each culture period was analyzed. Remaining single cells were eliminated from the analysis. For counting the cell number, an M-cluster was treated with trypsin for 15 min, followed by pipetting with a glass micropipette. The number of cells in each well was counted under a phase-contrast microscope. At least 20–30 M-clusters were analyzed at days 2, 3, 5, 7, 8, 11, and 14.

Method 11: RT-PCR. Naive cells (~10,000 cells per well of a 24-well plate), cells differentiated in vitro from single M-clusters (first and third generations) both from H-fibroblasts and H-MSCs (~10,000 cells per well of a 24-well plate), and SSEA-3^+/$CD105^+$ cells directly isolated from bone marrow aspirates were used. Total RNA was extracted from the cells and purified using NucleoSpin RNA XS (Macherey-Nagel). First-strand cDNA was generated using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. The PCR reactions were performed using Ex Taq DNA polymerase (TaKaRa Bio, Inc.). The used primers were as follows: β-actin sense: 5′-AGGCGGACTATGACTTAGTTGCGTTACACC-3′ and antisense: 5′-AAGTCCTCGGCCACATTGTGAACTTTG-3′, Nkx2.5 sense: 5′-GGGACTTGAATGCGGTTCAG-3′ and antisense: 5′-CTCATTGCACGCTGCATAATC-3′, α-fetoprotein sense: 5′-CCACTTGTTGCCAACTCAGTGA-3′ and antisense: 5′-TGCAGGAGGGACATATGTTTCA-3′, MAP-2 sense: 5′-ACTACCAGTTTCACACCCCCTTT-3′ and antisense: 5′-AAGGGTGCAGGAGACACAGATAC-3′, and GATA6 sense: 5′-CCTGCGGGCTCTACAGCAAGATGAAC-3′ and antisense: 5′-CGCCCCTGAGGCTGTAGGTTGTGTT-3′. Primers for Oct3/4 (Hs03005111_g1), Sox2 (Hs01053049_s1), and Nanog (Hs02387400_g1) were obtained from Applied Biosystems. Human whole embryo (Clontech) was used as a positive control for most PCR reactions, but human fetus liver (Clontech) was used for α-fetoprotein. In vitro differentiation of M-cluster-derived expanded cells into ectodermal (MAP-2), endodermal (α-fetoprotein and GATA6), and mesodermal (Nkx2.5) cells in RT-PCR (data shown in Fig. 2*F*) was confirmed with 20 different samples. The expression of these markers was reproduced in every M-cluster.

Method 12: Transplantation of Cells into Damaged Tissues. H-fibroblasts and H-MSCs were labeled with GFP-lentivirus, as previously reported (5), and examination under fluorescent microscopy confirmed that MEC populations or M-clusters generated from these GFP-labeled cells were positive for GFP.

After shaving and depilation, a skin defect (~13 mm^2), including the panniculus carnosus, was created on the backs of SCID mice (6). Cardiotoxin was injected into the cutaneous muscle of the SCID mice to induce muscle degeneration, as previously described (7). Carbon tetrachloride was administered to SCID mice by peritoneal injection to induce liver degeneration, as previously reported (8). For the skin wound model, 1×10^5 cells were transplanted by local injection into the edge of the wound 2 days after the injury. For damaged muscle and liver, 2×10^5 cells were transplanted by i.v. injection 2 days after inducing the damage. Either a GFP-labeled MEC population or GFP-positive SSEA3 (−) cells (sorted by FACS, as described below) were transplanted into the skin, muscle, and liver damage models, with three mice used for each condition. Intact mice that received i.v. injection of

either a GFP-labeled MEC population or GFP-labeled SSEA3(−) cells were used as controls. At 2 and 4 weeks after transplantation, mice were fixed with 4% (vol/vol) paraformaldehyde in 0.02 M PBS and subjected to immunohistochemistry and confocal laser microscopy, as described below.

Method 13: M-Cluster Formation from hBM Aspirates. Six hBM aspirates from healthy donors were purchased from ALLCELLS. Mononucleated cells were collected using Lymphoprep Tubes and subjected to MC culture at a concentration of 8×10^3 cells per microliter directly (naive hBM-MC) or after 8-hr LTT (8-hr hBM-MC), as described above. M-clusters were counted on day 7.

Method 14: MACS Sorting. All the antibodies used for MACS sorting were purchased from Miltenyi Biotech. Mononucleated cells isolated from hBM aspirates of three healthy donors (ALLCELLS) were first reacted with microbead-conjugated anti-CD105 antibody and sorted using MS Columns (Miltenyi Biotech) according to the manufacturer's instructions. CD105(+) cells were collected as fraction 1. CD105(−) cells were incubated with a mixture of anti-CD34 and anti-CD117 antibodies conjugated to microbeads and sorted again to obtain CD34(+)/CD117(+)/CD105(−) cells (fraction 2) and CD34(−)/CD117(−)/CD105(−) cells (fraction 3). The three samples were subjected to 8-hr LTT followed by MC culture, as described above. M-clusters were counted on day 7.

Method 15: Immunohistochemistry. Mouse testes were fixed with 4% (vol/vol) paraformaldehyde in 0.02 M PBS, and a cryostat was used to cut 10-μm-thick sections. For staining, samples were washed with 0.02 M PBS; incubated with 20% (vol/vol) Block-Ace (skim milk; Yukijirushi), 5% (vol/vol) BSA, and 0.3% Triton X-100 in 0.02 M PBS (blocking solution) at room temperature for 30 min; and incubated overnight at 4 °C with primary antibodies diluted in antibody diluent solution for immunohistochemistry (0.02 M PBS supplemented with 5% (vol/vol) BlockAce, 1% BSA, and 0.3% Triton X-100). The primary antibodies used were SMA (1:200; Lab Vision), neurofilament-M (1:200; Chemicon), α-fetoprotein (1:10; DAKO), human mitochondria (1:100; Abcam), dystrophin (1:100; Vector), human albumin (1:100; BETHYL Laboratories), α_1-antitrypsin (1:200; Ferma Scientific), Golgi complex (1:200; Abcam), cytokeratin 14 (1:200; Abcam), and Pax7 (1:200; DSHB, University of Iowa). After three washes with PBS, the slides were incubated with anti-rabbit IgG antibody conjugated with Alexa-488 or Alexa-568 (Invitrogen) or anti-mouse IgG antibody conjugated with Alexa-568 (Molecular Probes) in the presence of DAPI in the antibody diluents for 2 hr at room temperature. Samples were inspected with a C1si confocal microscope system (Nikon Corporation).

Method 16: Flow Cytometry and Cell Sorting. Cells were incubated with phycoerythrin-labeled antibodies against CD11c, CD29, CD34, CD44, CD45, CD49f, CD54, CD71, CD90, CD105, CD166, CD271, or von Willebrand factor (vWF; Becton Dickinson) or with anti-SSEA-3 antibodies (Millipore). In the case of labeling with the anti-SSEA-3 antibody, cells were further incubated with FITC-conjugated anti-rat IgM (Jackson Immunoresearch). Calcium and magnesium-free 0.02 M PBS supplemented with 2 mM EDTA and 0.5% BSA was used as the FACS antibody diluent. Data were acquired and analyzed using FACS (FACSCalibur; Beckton Dickinson) and CellQuest software (Beckton Dickinson) or using FACS (FACSAria; Becton Dickinson) and DIVA software (Becton Dickinson). For cell sorting, cells were incubated with anti-SSEA-3 (detected by FITC) and anti-CD105 (detected by Alexa-647) in the FACS antibody diluents and sorted by the FACSAria using a low stream speed to ensure a high level of cell survival and in the four-way purity sorting mode to obtain the highest purity of the sorted cells.

Method 17: Statistical Analysis. Data were compared using ANOVA, with pairwise comparisons using the Bonferroni method.

Method 18: Miscellaneous. For immunocytochemistry and FACS experiments of MEC populations, trypsin was neutralized after LTT by the addition of serum. The cells were centrifuged, and the medium was replaced with 10% (vol/vol) FBS in α-MEM. Before performing the above experiments, the cells were incubated for 3 hr at 37 °C to allow protein synthesis to recover. Furthermore, for immunocytochemistry, testis injection, and FACS experiments, samples after LTT were filtered with Cell Strainer (Φ100 μm; BD Falcon) to remove the genomic DNA from dead cells, which would have interfered with the experiments.

SI Results

Result 1: Stress Conditions for H-Fibroblasts and H-MSCs. After exposing the cells to stress conditions and vortexing, trypan blue staining was used to count the number of live cells, from which the survival ratio was calculated. The surviving cells were collected and grown in MC culture for 7 days. Stress condition *ii* resulted in a large number of dead cells, which had a toxic effect on the few surviving cells. It was therefore not possible to determine accurately the number of formed M-clusters, and the number of M-clusters for stress condition *ii* is thus denoted as not determined in Table S1.

Among the six stress conditions tested, the 16-hr trypsin incubation was most effective for the formation of M-clusters for H-fibroblasts and the 8-hr trypsin incubation was most effective for H-MSCs. When this experiment was repeated using two strains of H-fibroblasts and four strains of H-MSCs, the same trend was observed. M-clusters could not be recognized in the negative control using human peripheral mononuclear cells. An example of observed value is shown in Table S1.

The series of manipulations, namely, a 16-hr or 8-hr incubation with trypsin followed by vortexing at 1,800–2,200 rpm/min by MS1 Minishaker (IKA Works, Staufen, Germany). for 3 min and centrifugation at $740 \times g$ for 15 min, was termed "long-term trypsin incubation" and used for the enrichment of Muse cells. Collection efficiency of live cells after vortexing was 70–80%.

Result 2: Criteria for M-Clusters. The average diameter of cells in MEC populations from both H-fibroblasts and H-MSCs was 10–13 μm (Fig. S1*A*). When these cells were transferred to MC culture, the cells started to proliferate. The size of the individual cells became smaller after cell division, and the gradually forming multicellular clusters comprised cells 8–10 μm in diameter. The size and appearance of the cells were similar to those of cells in clusters formed by hES cells (Fig. S1*B*, generated in MC culture, day 3). At day 7, most of the multicellular clusters larger than 25 μm had an appearance very similar to cell clusters formed by hES cells (Fig. S1*C*). Cell clusters derived from hES cells were positive for ALP staining (Fig. S1*D*, day 4 in MC culture). Using 25-μm filters, we isolated clusters derived from both H-fibroblasts and H-MSCs that were larger than 25 μm and analyzed 100 clusters each by immunocytochemistry. Most of the clusters contained cells positive for the pluripotency markers, such as Oct3/4, Sox2, and PAR4 (Fig. S1 *E*–*G*), and for ALP staining, as in the case of Fig. 1*K*. Pluripotency markers could also be detected in clusters smaller than 25 μm, but their localization was sometimes atypical and the appearance of the cells was more similar to those of MEC populations. Furthermore, the results of ALP staining were ambiguous. Based on these findings, only multicellular clusters larger than 25 μm were counted as M-clusters.

Result 3: Karyotype Analysis. Karyotypes of cells expanded from M-clusters derived from both H-fibroblasts and H-MSCs did not show detectable abnormalities (Fig. S4).

Result 4: Injection of Cells into the Testes of Immunodeficient Mice. Within 8 weeks, all mice injected with mouse ES cells developed

teratomas but not those injected with MEF cells. In 10 of 13 testes injected with MEC populations and in 10 of 11 testes injected with M-clusters, cells positive for human mitochondria, neurofilament, α-fetoprotein, and SMA were detected.

Result 5: Transplantation of Cells into Damaged Tissues. Transplanted GFP-labeled MEC populations or GFP-positive SSEA3 (−) cells were not detected in the host brain, heart, intestine, liver, kidney, or muscles of intact control mice (4 weeks). In the lung, however, a small number of GFP-positive transplanted cells were detected. Furthermore, when the brain, heart, intestine, kidney, and muscles of liver-damaged mice were examined 4 weeks after transplantation, GFP-labeled transplanted cells were not detected in these intact tissues. These findings indicate that transplanted cells incorporate mainly into damaged tissues.

Result 6: MACS Sorting. The three fractions from MACS sorting of mononucleated cells from bone marrow contained the following percentages of total cells: (*i*) $CD34^-/CD117^-/CD105^+$ fraction, 1.8%; (*ii*) $CD34^+/CD117^+/CD105^-$ fraction, 8.5%; and (*iii*) $CD34^-/CD117^-/CD105^-$ fraction, 89.7%. The frequencies of M-cluster formation in *i*, *ii*, and *iii* were 0.5 ± 0.03%, 0%, and 0.01 ± 0.002%, respectively. The formation of M-clusters in the $CD34^-/CD117^-/CD105^+$ fraction was thus ∼50 times higher than in the $CD34^-/CD117^-/CD105^-$ fraction. This trend was reproducible in three independent experiments.

Result 7: FACS of Naive Cells and MEC Population. FACS analysis revealed that naive H-fibroblasts and H-MSCs express high levels of CD29, CD44, CD54, CD90, and CD105, all of which are markers specific for mesenchymal cells (7, 9); low to moderate levels of CD49f, CD71, CD166, CD271, and vWF; and no CD11c, CD34, or CD45. MEC populations expressed low levels of CD29, CD44, CD49, CD54, CD71, CD166, CD271, and vWF and moderate levels of CD90 and CD105 but showed a substantially increased number of SSEA-3-positive cells [naive cell populations contained 1.1 ± 0.05% (H-MSCs) and 1.8 ± 0.22% (H-fibroblasts) of SSEA-3-positive cells, which increased to 11.6 ± 0.15% (H-MSCs) and 8.6 ± 0.0.32% (H-fibroblasts) after LTT].

In both H-fibroblasts and H-MSCs, SSEA-3(+) and SSEA-3(−) cells were separated by FACS sorting and subjected to single-cell suspension culture. A total of 56.5 ± 3.2% (H-MSCs) and 60.0 ± 4.5% (H-fibroblasts) of the SSEA-3(+) cells generated M-clusters, whereas only a few M-clusters formed from SSEA-3(−) cells in both H-MSCs and H-fibroblasts. If SSEA-3 expression is a defining property of Muse cells, M-cluster formation in the SSEA-3(+) cells is expected to be very high. It is conceivable that the sorting process caused cell damage, somewhat reducing the number of M-clusters that formed. In SSEA-3(−) cells, only a few of the cell clusters were larger than 25 μm. These results thus suggest that SSEA-3 expression is closely related to the identity of Muse cells.

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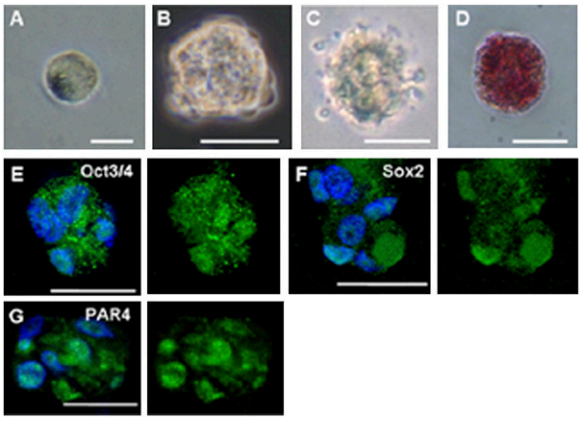

Fig. S1. (*A*) Individual cell in an MEC population. (Scale bar: 10 μm). (*B*) hES cell-derived M-cluster at day 3. (Scale bar: 25 μm.) (*C*) M-cluster at a size of ∼25 μm. (Scale bar: 25 μm.) (*D*) ALP staining of an hES cell-derived cell cluster at day 4. (Scale bar: 25 μm.) Immunocytochemistry of small M-clusters showing the localization of Oct3/4 (*E*), Sox2 (*F*), and PAR4 (*G*). The pluripotency markers were labeled with Alexa-488 (green), and all samples were counterstained with DAPI (blue). (Scale bars: 25 μm.)

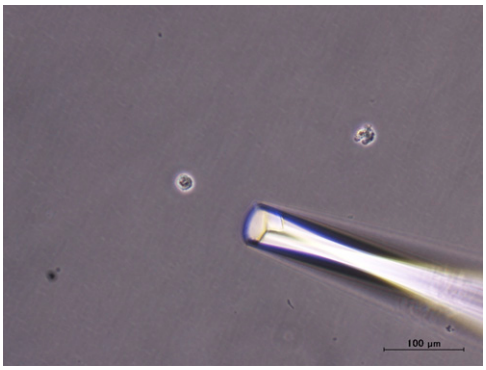

Fig. S2. Picking up a single cell using a glass micropipette. (Scale bar: 100 μm.)

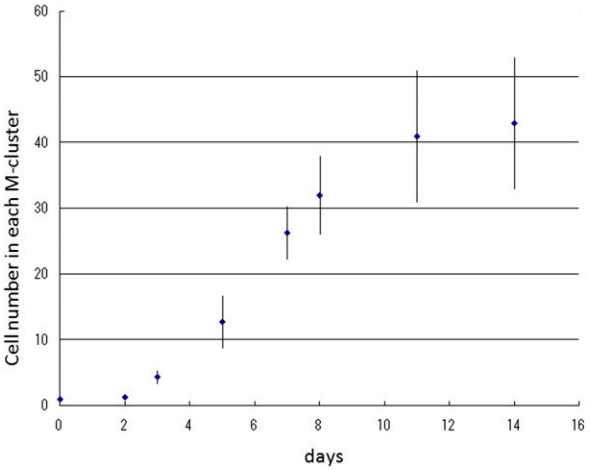

Fig. S3. Cell growth of Muse cells (H-fibroblasts) in MC culture.

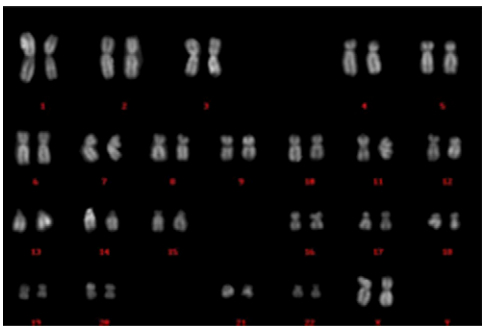

Fig. S4. Normal karyotype of cells expanded from a single M-cluster (H-fibroblast-1, first generation).

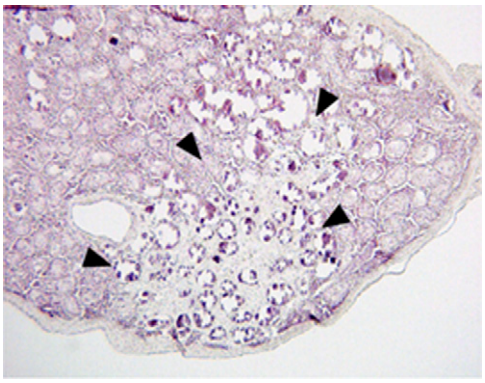

Fig. S5. Histology of M-cluster (H-fibroblast) injected in immunodeficient mouse testis at 6 months (H&E staining). The area indicated by arrowheads is the site of integration.

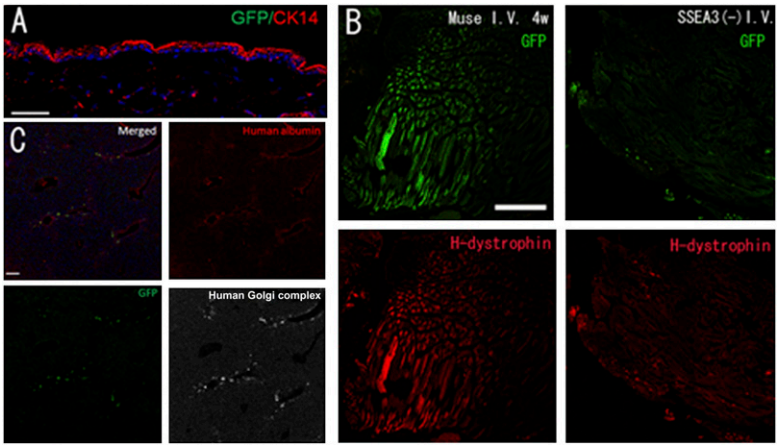

Fig. S6. Transplantation of GFP-positive SSEA3(–) cells into skin (*A*), muscle (*B*), and liver-damaged model mice (*C*) at 4 weeks. (*A*) Compared with the GFP-labeled MEC population, few GFP-positive transplanted cells were observed in the epidermis of regenerating skin. (*B*) A small number of human (H) dystrophin (+)/GFP-labeled transplanted cells were observed in the GFP-positive SSEA3(–)-cell transplanted group (*Right*), whereas there were significantly more human dystrophin(+)/GFP-labeled transplanted cells in the MEC population-transplanted mice (*Left*). (*C*) Only a small number of human albumin(+)/human Golgi complex(+)/GFP-labeled transplanted cells were observed compared with the MEC population-transplanted group (see main text). (Scale bars: *A*, 50 μm; *B*, 1 mm; *C*, 100 μm.)

Table S1. An example of ratios of survival after stress and cell cluster formation in MC culture in H-fibroblasts, H-MSCs, and human peripheral mononuclear cells

		Start cell number	Survival after stress (%)	Cell cluster formation in MC culture (>25 μm) (% of surviving cells)
H-fibroblast-1				
1	Nonserum	30,000	75	7
2	HBSS	2,000,000	6	ND
3	10% FBS + low O_2	30,000	99	8
4	Trypsin, 3 × 1-hr washes	2,000,000	0.3	6
5	LTT, 8 hr	2,000,000	1	15
6	LTT, 16 hr	500,000	5	20
H-MSC-1				
1	Nonserum	30,000	44	5
2	HBSS	2,000,000	2	ND
3	10% FBS + low O_2	300,000	99	8
4	Trypsin, 3 × 1-hr washes	380,000	0.9	9
5	LTT, 8 hr	380,000	10	21
6	LTT, 16 hr	500,000	3	14
Human peripheral mononuclear cells				
5	LTT, 8 hr	300,000	2	0
6	LTT, 16 hr	300,000	1	0

ND, not determined; cell clusters could not be calculated accurately because the final fraction contained a large number of dead cells.