SUPPLEMENTAL MATERIAL

Detailed Materials and Methods

Animal Model

All animals were treated in accordance with the Code of Ethics of the World Medical Association as well as Tohoku University guidelines based on the International Guiding Principles for Biomedical Research Involving Animals, and the animal protocols were approved by Tohoku University's Administrative Panel on Laboratory Animal Care.

Lacunar infarction was induced by previously reported method[1]. Male severe combined immunodeficiency (SCID) (8-10w) mice were anesthetized with 1.5% isoflurane and mounted by stereotaxic apparatus (SR-5M, NARISHIGE, Japan). Two vasoconstrictive peptide, endothelin-1 (ET-1) and N(G)-nitro-L-arginine methyl ester (L-NAME), were stereotaxically co-injected into posterior limb of internal capsular (from the bregma: anterior-posterior (AP), -2.0 mm; medial-lateral (ML), +2.0 mm; dorsal-ventral (DV, from dural surface), -4.0 mm). 8 weeks after, demyelination was histologically confirmed at the injected site (Figure 1A). Neuron tracing with dextran, an anterograde tracer, was used to assess axonal interruption. Eight weeks after stroke, dextran conjugated Alexa Flour 594 (D-22913, Invitrogen, USA) was injected into the motor cortex, and dextran positive signal was examined at cervical spinal cord C1-2 level 1 week after the injection for validation of axonal interruption (Figure 1B).

Preparation of Human Muse Cells

Human Muse cells were separated from human BM-MSCs (Lonza Inc., Allendale, NJ, USA) which were cultured in α-minimum essential medium (α-MEM) with 10% (vol/vol) fetal bovine serum (FBS), as previously described[2,3]. In this experiment, BM-MSCs from the 6th to 8th subculture were used, and for labeling, they were introduced with lentivirus-GFP prior to FACS isolation, as described[4]. Then, Muse cells were isolated by FACS (FACS Aria 2, Becton Dickson, Franklin Lakes, NJ, USA) using an anti-SSEA-3 antibody detected by fluorescein isothiocyanate (FITC)[2,3]. Animals transplanted with FACS-sorted Muse cells were mainly used for determining mechanism of action via histological assays (i.e., cell distribution in the brain, immunohistological staining and anterograde tracing of pyramidal tracs).

In parallel experiment, animals that received MACS-sorted serum/xeno-free Muse-rich cells were prepared for efficacy (i.e., behavioral analysis using corner turn and cylinder tests) and safety validation. Particularly for the latter purpose, Muse cells used for this experiment were unlabeled. 'Serum/xeno-free human MSCs' were purchased from Gibco (StemPro BM Mesenchymal Stem Cells, A15652, Gibco, life technologies, CA, USA) and were cultured in xeno-free medium (StemPro MSC SFM, A1033201, life technologies). Then, Muse cells were sorted from serum/xeno-free human MSCs by MACS (autoMACS Pro Separator, Miltenyi Biotec, Germany) by using SSEA-3 antibody (1:100; MAB4303, Millipore, Temecula, CA) which is detected by FITC and anti-FITC microbeeds (1:10, 130-048-075, Miltenyi Biotec, Germany). The positive fraction was defined as Muse-rich cells (Muse-rich) and subjected to transplantation.

Characterization of Human Muse Cells

Single-cell suspension of FACS-isolated Muse cells was performed for 7 days in order to check single cell-derived cluster formation, as previously described[2]. The formed clusters were then individually transferred onto gelatin coated 4-well dishes, allowing cells to expand from the adhered cluster on gelatin. Two weeks after, cells were fixed by 4% paraformaldehyde in phosphate buffered saline (PBS) and stained either with anti-neurofilament (1:200; AB1987, Millipore), anti-

cytokeratin 7 (1:100; MAB3226, Millipore) and anti-smooth muscle actin (1:200; MS-113-P0, Thermo-Fisher Scientific). Neural spheres formed from serum/xeno-free Muse cells were generated under the neural induction medium (Neurobasal Medium (21103-049, life technologies) containing B-27, GlutaMAX, epidermal growth factor (30 ng/ml) and basic-fibroblast growth factor (30 ng/ml) for 1 week, as previously described [12]. Generated neural spheres were stained with the neural stem cells markers NeuroD (1:100; ab60704, Abcam), Sox2 (1:500; ab59776, Abcam), nestin (1:100; MAB5326, Millipore) and Musashi-1 (1:1000; AB5977, Millipore). Samples were inspected under laser confocal microscope (C2si; Nikon, Tokyo, Japan).

Cell Transplantation

Two weeks after induction of lacunar infarction by ET-1/L-NAME[1], either human Muse cells (FACS isolated GFP-labeled Muse group (n=12)/ MACS-sorted Muse-rich group (n=13)) or serum/xeno-free MSCs as 'MSC group' (n=7) were stereotaxically transplanted into the peri-lesion (from bregma: AP, -2.0 mm; ML, +2.0 mm; DV, -3.0 mm) because likely none of the transplanted cells would have survived if they were directly implanted to the necrotic core. The number of transplanted cells was 100,000 diluted with 3 μl PBS. The same volume of PBS (n=7) (vehicle group) was transplanted as control groups.

Histologic Analysis

Eight weeks after transplantation of either the FACS-isolated GFP-Muse cells, MACS-sorted Muse-rich cells, MSCs or vehicle, mice were anesthetized and perfused intracardially with PBS followed by periodate lysine paraformaldehyde (PLP) solution (0.01 M $NaIO_4$, 0.075 M lysine, 2% paraformaldehyde, pH6.2). Tissues were postfixed for 6 hours in the same fixative at 4°C. Fixed tissues were cryoprotected by immersing into 15%, 20% and 25% sucrose overnight at 4°C, embedded in O.C.T. compound (25608-930, Sakura Finetek USA, Inc., USA) and were cut into 7 μm thick sections using a cryostat (CM1850; Leica, Wetzlar, Germany). Sections were stained with hematoxylin and eosin (H&E) and luxol fast blue. The presence of engrafted cells in the host brain and evaluation of their differentiation into neural-lineage were assessed by fluorescent immunohistochemistry using antibodies against human mitochondria (hMit)(1:100; ab3298, Abcam), human golgi complex (1:100; ab27043, Abcam), GFP (1:1000; ab6673, Abcam), NeuN (1:200; MAB377, Millipore), microtubule-associated protein-2 (MAP2) (1:1000; M1406, Sigma), GST-pi (1:500; 312, MBL), glial fibrillary acidic protein (GFAP) (1:500; IR524, DAKO), Iba-1 (1:500; ab5076, Abcam), Ki67 (1:100; ab15580, Abcam), synaptophysin (1:1000, MAB5258, Millipore) and vesicular glutamate transporter (VGluT) (1:200; 821301, BioLegend). The samples were then incubated either with anti-mouse IgG, anti-rabbit IgG and anti-goat IgG secondary antibodies conjugated with alexa-488 or -568, counter stained with 4',6-diamidino-2-phenylindole (DAPI)(1:1000; D9542, Sigma) and inspected under laser confocal microscope (C2si; Nikon).

Neuronal Tracing Analysis

A neuron tracing study was performed as previously described with some modifications[5]. Dextran conjugated Alexa Flour 594 (D-22913, Invitrogen, USA) was used as anterograde tracer. For detecting host pyramidal tract, tracer was stereotactically injected at these coordinates: from bregma (1) AP, 0 mm; ML, +1.0 mm; DV, -0.5 mm; (2) AP, +0.5 mm; ML, +1.5 mm; DV, -0.5 mm; (3) AP, +1.0 mm; ML, +1.5 mm; DV, -0.5 mm. These targets approximated the motor cortex (M1 area). For tracing extended neurites of transplanted FACS-isolated Muse cells, dextran tracer

was stereotactically injected into the lesion site (from bregma: AP, -2.0 mm; ML, +2.0 mm; DV, -3.0 mm). Tissue sections were prepared one week after the injection, as described above.

Behavioral Analysis

The neurological deficit was evaluated with corner turn and cylinder tests[6,7]. Behavioral assessments were performed at -2 (before lacunar infarct) and 0 weeks, and 2, 4, 6, 8 weeks after transplantation of the MACS-sorted Muse-rich cells, MSCs and vehicle by an investigator blinded to the experimental group. After the follow-up period, loss of functional study using diphtheria toxin (DT) was performed in the Muse and vehicle groups. Rodent cells are 100,000 times less sensitive to DT compared with human cells[8], and DT has been used as a tool for targeted ablation of human cells in rodent models[9]. At 8 weeks after transplantation, mice received intraperitoneal injections of DT (50 μg/kg) twice at 24 h interval and were reassessed behaviorally at 1week after DT administration. DT was administered in a separate cohort of animals with lacunar infarct and transplanted with MACS-sorted Muse-rich cells or vehicle.

Q-PCR for Detecting Human-specific Alu Sequence

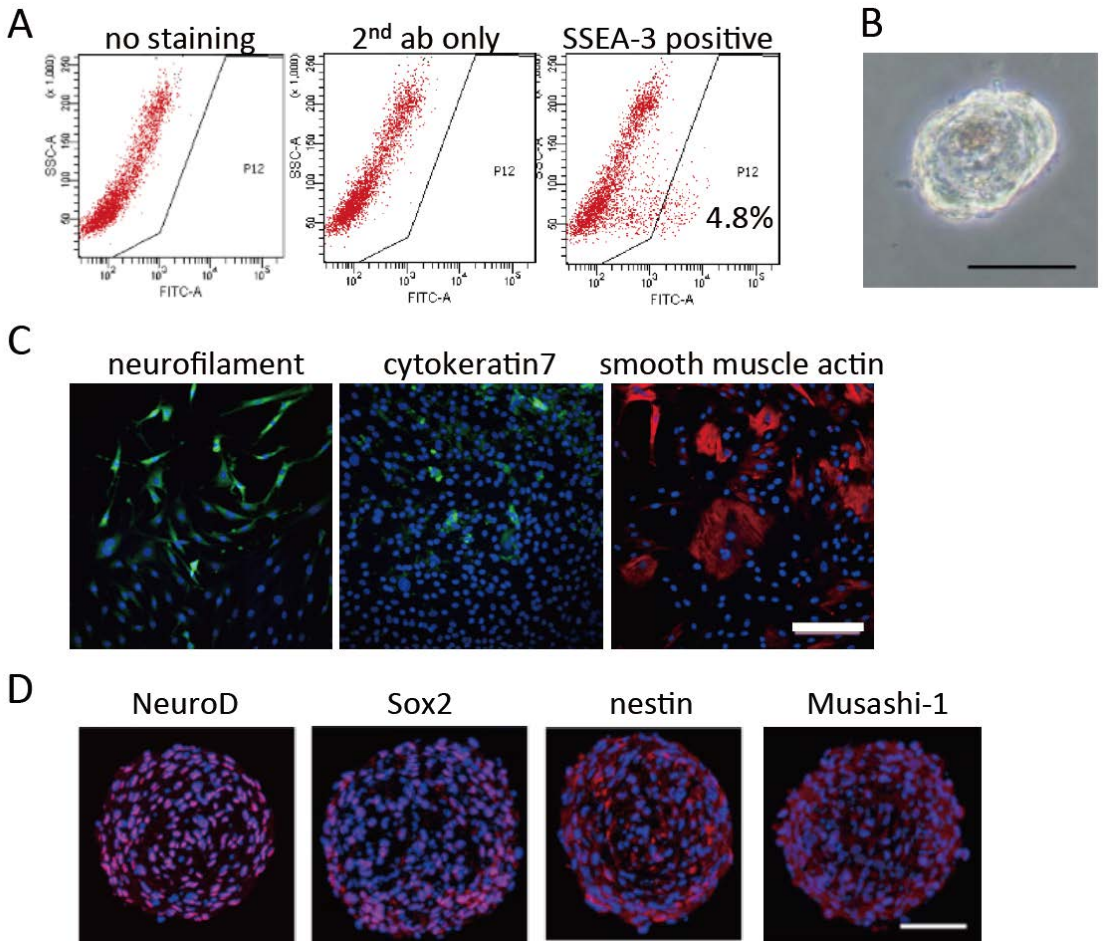
DNA was extracted from the brain, lung, liver, spleen and kidney of each animal in the MACS-sorted Muse-rich group 6 months after transplantation and in intact healthy SCID mice 10 months after receiving MACS-sorted Muse-rich cells transplantation at the same position. DNA concentration was arranged 20 ng/ml. The samples were applied to Applied Biosystems 7500 (life technologies, USA) for quantitative-polymerase chain reaction (Q-PCR). Q-PCR was consisted of 50°C for 2 min and 95°C for 10 min followed by 50 cycles of 95°C for 15s, 58°C for 30s, 72°C for 30s. The amount of human Alu sequence was calculated from the calibration curve. The Alu PCR primer and TaqMan probe[10] are available from the authors.

Statistics

Data were expressed mean ± standard deviation (SD). Statistical analysis was performed with GraphPad Prism 5 (MDF, Japan), with behavioral, histological, and Q-PCR data assessed using repeated measures ANOVA, followed by Bonferroni posthoc tests.

ONLINE SUPPLEMENT

Supplemental Figure I



Supplemental Figure I. Characterizations of serum-/xeno-free Muse cells in vitro. Muse cells, positive for specific embryonic antigen SSEA-3, were isolated with fluorescence-activated cell sorter (FACS) (**A**). A representative cluster generated from serum-/xeno-free Muse cells under the single suspension when cultured for 1 week (**B**). The cells expanded from adherent clusters on gelatin-coated dish for 2 weeks contained cells positive for triploblastic lineage markers; neurofilament (ectoderm), cytokeratin7 (mesoderm), smooth muscle actin (endoderm) (**C**). The neural spheres generated from serum-/xeno-free Muse cells under the condition of neural stem cell induction for 1 week were positive for neural stem cell markers; NeuroD1, Sox2, nestin and Musashi-1 (**D**). Scale bar in **B**, 50 μm. Scale bar in **C** and **D**, 100 μm.

References

1. Uchida H, Sakata H, Fujimura M, Niizuma K, Kushida Y, Dezawa M, et al. Experimental model of small subcortical infarcts in mice with long-lasting functional disabilities. *Brain Res*. 2015;1629:318-328
2. Kuroda Y, Kitada M, Wakao S, Nishikawa K, Tanimura Y, Makinoshima H, et al. Unique multipotent cells in adult human mesenchymal cell populations. *Proc Natl Acad Sci U S A*. 2010;107:8639-8643
3. Kuroda Y, Wakao S, Kitada M, Murakami T, Nojima M, Dezawa M. Isolation, culture and evaluation of multilineage-differentiating stress-enduring (muse) cells. *Nat Protoc*. 2013;8:1391-1415
4. Hayase M, Kitada M, Wakao S, Itokazu Y, Nozaki K, Hashimoto N, et al. Committed neural progenitor cells derived from genetically modified bone marrow stromal cells ameliorate deficits in a rat model of stroke. *J Cereb Blood Flow Metab*. 2009;29:1409-1420
5. Hellenbrand DJ, Kaeppler KE, Hwang E, Ehlers ME, Toigo RD, Giesler JD, et al. Basic techniques for long distance axon tracing in the spinal cord. *Microsc Res Tech*. 2013;76:1240-1249
6. Zhang L, Schallert T, Zhang ZG, Jiang Q, Arniego P, Li Q, et al. A test for detecting long-term sensorimotor dysfunction in the mouse after focal cerebral ischemia. *J Neurosci Methods*. 2002;117:207-214
7. Li X, Blizzard KK, Zeng Z, DeVries AC, Hurn PD, McCullough LD. Chronic behavioral testing after focal ischemia in the mouse: Functional recovery and the effects of gender. *Exp Neurol*. 2004;187:94-104
8. Pappenheimer AM, Jr., Harper AA, Moynihan M, Brockes JP. Diphtheria toxin and related proteins: Effect of route of injection on toxicity and the determination of cytotoxicity for various cultured cells. *J Infect Dis*. 1982;145:94-102
9. Cummings BJ, Uchida N, Tamaki SJ, Salazar DL, Hooshmand M, Summers R, et al. Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. *Proc Natl Acad Sci U S A*. 2005;102:14069-14074
10. Nicklas JA, Buel E. Development of an alu-based, real-time pcr method for quantitation of human DNA in forensic samples. *J Forensic Sci*. 2003;48:936-944