Human Muse Cells Reconstruct Neuronal Circuitry in Subacute Lacunar Stroke Model

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Background and Purpose—Multilineage-differentiating stress-enduring (muse) cells are endogenous nontumorigenic stem cells with pluripotency harvestable as pluripotent marker SSEA-3+ cells from the bone marrow from cultured bone marrow-mesenchymal stem cells. After transplantation into neurological disease models, muse cells exert repair effects, but the exact mechanism remains inconclusive.

Methods—We conducted mechanism-based experiments by transplanting serum/xeno-free cultured-human bone marrow-muse cells into the perilesion brain at 2 weeks after lacunar infarction in immunodeficient mice.

Results—Approximately 28% of initially transplanted muse cells remained in the host brain at 8 weeks, spontaneously differentiated into cells expressing NeuN (≈62%), MAP2 (≈30%), and GST-pi (≈12%). Dextran tracing revealed connections between host neurons and muse cells at the lesioned motor cortex and the anterior horn. Muse cells extended neurites through the ipsilateral pyramidal tract, crossed to contralateral side, and reached to the pyramidal tract in the dorsal funiculus of spinal cord. Muse-transplanted stroke mice displayed significant recovery in cylinder tests, which was reverted by the human-selective diphtheria toxin. At 10 months post-transplantation, human-specific Alu sequence was detected only in the brain but not in other organs, with no evidence of tumor formation.

Conclusions—Transplantation at the delayed subacute phase showed muse cells differentiated into neural cells, facilitated neural reconstruction, improved functions, and displayed solid safety outcomes over prolonged graft maturation period, indicating their therapeutic potential for lacunar stroke. (Stroke. 2017;48:428-435. DOI: 10.1161/STROKEAHA.116.014950.)

Key Words: brain ischemia ■ cerebral infarction ■ regeneration ■ stem cells ■ stroke, lacunar

Lacunar infarcts account for ≈25% of all ischemic stroke.1 Patients with lacunar infarcts normally present with a good vital prognosis because of small lesion size. However, when the pyramidal tract is involved in the lesion, the functional outcome of lacunar infarct patients is always unfavorable, irrespective of the size of the lesion, and often associated with long-lasting motor disabilities.2,3 Although tPA (tissue-type plasminogen activator) is reported to confer functional recovery in acute phase (within 4.5 hours after onset) ischemic stroke,4 rehabilitation is primarily the only option beyond this narrow therapeutic window of tPA poststroke. Such unmet clinical need has warranted novel approaches for ischemic stroke, with stem cell therapy emerging as an experimental stroke therapeutic in recent years. Mesenchymal stem cells (MSCs) are considered pertinent to clinical use because they are nontumorigenic, easily accessible from donor tissue sources, such as banked bone marrow (BM), do not involve ethical problems and are expandable to clinical scale. The postulated mechanism of action involves trophic factor secretion, with replenishment of new functional cells not well documented, owing in part to poor homing rate of MSCs into damaged tissue after transplantation.5 Preclinical and clinical studies have reported mixed outcomes in grafted MSCs, with mediocre transplant survival in the host tissue and inconsistent efficacy in the long term.6-9 To this end, it is desirable to cater stem cell therapy toward affording cell replenishment to the injured host brain, where transplanted cells may integrate with the stroke brain in affording robust and stable functional recovery.10 In an effort to test this hypothesis, we posit that the lacunar infarcts serve as a good disease platform...
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for assessment of stem cell graft-mediated cell replenishment mechanism because the lesion is highly confined to the white matter whose structure is homogenously composed of neurons and myelinating oligodendrocytes.

Multilineage-differentiating stress-enduring (muse) cells are a novel type of endogenous stem cells that are able to self-renew, display pluripotency, and differentiate into cells representative of all three germ layers from a single cell and tolerate stresses. They reside in the nontumorigenic mesenchymal tissues such as the BM, adipose tissue, and dermis,11 expressing the pluripotent surface marker, stage specific embryonic antigen (SSEA)-3.11–13 The proportion of muse cells in the BM-mononucleated cells is ≈0.03%, so that ≈30 mL BM aspirate yields approximately 1 million muse cells by ≈3 days.11 Intravenously injected naive muse cells migrate to and integrate into damaged sites and spontaneously differentiate into functional cells in injury models of the liver, muscle and skin.11,14 Grafted muse cells contribute to tissue reconstruction in skin ulcers of a diabetes mellitus model by replenishing new dermal and epidermal cells; similarly human skin fibroblast-derived muse cells rebuild pyramidal and sensory tracts by replacing new functional neuronal cells in the cortex that could extend neurites into the contralateral spinal cord and physiologically evoke firing potentials.15–17 Unlike embryonic stem (ES) and induced pluripotent stem (iPS) cells, naive muse cells require neither introduction of exogenous genes for reprogramming cells nor implementation of cytokine induction protocol to make them lineage-committed cells, including their spontaneous differentiation into functional neuronal cells after homing into the damaged brain, indicating their suitability as donor cells for transplantation in neurological disorders, such as stroke.11–13,17

In this study, with the goal of translating human BM-muse cells for clinical use, we used serum- and xeno-free cell culture system in preparing muse cells for transplantation into the perilesion brain of immunodeficient mice at subacute phase of lacunar infarction. Initially, we performed fluorescence-activated cell sorter (FACS), which can purify GFP (green fluorescent protein)–labeled muse cells, thereby allowing us to accurately ascribe functional and histological outcomes of transplantation to the muse cells. We also prepared magnetic-activated cell sorter (MACS)–isolated muse cells, whereas MACS neither achieves 100% purity nor allows collecting GFP(+)–muse cells, cytotoxicity is moderate and final collection efficiency is generally higher than FACS. Furthermore, MACS-sorted cells have already been applied in a clinical study.18 Because cells other than the targeted muse cells may contaminate the positive fraction, we realized that data generated from MACS-sorted cells may not be an outcome of pure muse cells. The individual drawbacks by FACS and

Figure 1. Generation of lacunar infarct model and preparation of muse cells sorted by magnetic-activated cell sorter (MACS). Lacunar infarct was made by administration of 2 vasoconstrictive peptides. Eight weeks after, demyelination (arrow heads) in ipsilateral side was reconfirmed by luxol fast blue staining (A), and the axonal interruption was also reconfirmed by dextran tracing at cervical spinal cord C1-2 level (red signal; B). Analysis of SSEA-3–positive cells before and after MACS sorting is shown in C. The positive fraction containing >70% muse cells was used for transplantation as muse-rich cells. The negative fraction contained muse cells at ≈0.6%. Scale bar, 1000 μm (A) and 100 μm (B). DAPI indicates 4',6-diamidino-2-phenylindole; ET-1/L-NAME, endothelin-1/ N(omega)-nitro-L-arginine methyl ester; and FITC, fluorescein isothiocyanate.
MACS therefore guided us to use FACS-isolated muse cells for mechanism-based investigations, whereas for translational application, we assessed efficiency and safety MACS-sorted muse cells.

Materials and Methods

Animal Model
All animals (male severe combined immunodeficiency; CB17/Icr-Pkrdcsdcsd/Prkdc<scid>/CrlCrlj at 8–10 weeks) 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. The same size of lacunar infarction was induced using previously reported method19 (Figure 2A).

Preparation of Human Muse Cells
Human muse cells were separated from human BM-MSCs as previously described11,13 and processed either by FACS isolation20 or MACS sorted. Non-muse SSEA-3(-) cells served as controls.

Cell Transplantation
Two weeks after induction of lacunar infarction by ET-1/L-NAME (endothelin-1/N(omega)-nitro-L-arginine methyl ester),19 100,000 FACS-isolated GFP-labeled muse cells, MACS-sorted muse-rich cells, or serum/xeno-free MSCs as MSC were stereotaxically transplanted into the perilesion site. Infusion of the vehicle PBS with the same volume served as control groups. For behavior analysis, we prepared 7 animals per group for of the MACS-sorted muse-rich, MSC, and vehicle groups. For histological analysis that included anterograde tracing experiment, we prepared 6 and 5 animals for the FACS-sorted muse and MACS-sorted muse-rich groups, respectively. Of note, using different cell selection methods for multiple experiments may induce a risk for methodological and qualitative biases, which should be addressed by incorporating proper controls as pursued in this study.

Statistics
Data were expressed mean±SD. Statistical analysis was performed with GraphPad Prism 5 (MDF, Japan), with behavioral, histological, and quantitative polymerase chain reaction data assessed using repeated-measures ANOVA, followed by Bonferroni post hoc tests. Defined Methods are available in the online-only Data Supplement.

Results

Differentiation Ability of Muse Cells Into Neural Lineage Cells
Muse cells when grown in suspension formed characteristic clusters from a single cell. These clusters resembled embryonic stem cell–derived embryoid bodies. On the contrary, none of such clusters were formed from nonmuse cells grown in suspension (Figure 1A in the online-only Data Supplement).11–13,21 These muse cells spontaneously differentiated into triploblastic lineages: neurofilament (ectodermal, 2.6±1.1%), cytokeratin7 (endodermal, 2.1±1.5%), and smooth muscle actin (mesodermal, 16.8±2.4%; Figure 1B in the online-only Data Supplement). Under neural stem cell induction medium, muse cells formed neural spheres that contained cells positive for neural stem cell markers NeuroD1, Sox2, Nestin, and Musashi-1 (Figure 1C in the online-only Data Supplement). These results indicated that the basic property of muse cells was not altered by serum/xeno-free culture system,11–13,21 and that single cell–derived clusters reflect triploblastic and neural differentiation potentials of muse cells.

MSCs, which originally contained 3.2±0.79% of SSEA-3+ muse cells, yielded the positive fraction with 71.3±0.65% of muse cells after MACS separation (Figure 1C). In negative fraction, the proportion of muse cell was 0.6±0.08% (Figure 1C). MACS-sorted muse-rich cells generated cluster formation from a single cell in suspension, reminiscent of triploblastic differentiation of the single cell–derived cluster, as described above (not shown). Furthermore, under neurosphere induction, muse-rich cells formed neurosphere-like...
clusters positive for NeuroD1, Nestin, Sox2, and Musashi-1, as described above (not shown). These results indicated that the phenotype of muse cells contained in the muse-rich cells after MACS sorting did not differ from FACS-isolated muse cells.

**Distribution of Transplanted Muse Cells Within the Host Infarcted Brain**

At 8 weeks after transplantation, few MSCs were detected in the brain (not shown), whereas many FACS-isolated GFP-labeled muse cells populated the region between −1 and −3 mm from the bregma. Mean numbers of muse cells per slice were 21±2.2, 82.3±13.7, and 23.3±5.2 cells at −1, −2, and −3 mm, respectively (Figure 2A), indicating that most transplanted muse cells remained proximal to the lesion site (Figure 2B, top). A similar graft distribution was detected in transplanted MACS-sorted muse-rich cells (Figure 2B, bottom). The total number of grafted cells that integrated into the host brain was ≈28% of initially injected muse cells.

**Neural Differentiation of Transplanted Muse Cells**

Whereas FACS-sorted GFP-muse cells could not be detected in contralateral part of the brain (not shown), several muse cells were observed within 400-μm area from the ipsilateral transplanted site (Figure 2A) expressing NeuN (neuronal marker), MAP2 (neuronal marker), and GST-pi (oligodendrocytic marker; Figure 3A). Positivity for NeuN was 62.2±2.4% of GFP-positive muse cells, MAP2 30.6±3.1%, and GST-pi 12.1±1.1% (Figure 3B). GFAP (astrocytic marker), Iba-1 (microglial marker), and Ki67 (marker for proliferating cells) could not be detected in GFP-muse cells (Figure 3C). GFP-labeled muse cells were further shown to be positive for human golgi complex and human mitochondria, suggesting their human origin (Figure 3D). In the brain sections of MACS-sorted muse-rich cells, human golgi complex-positive muse cells expressed NeuN, suggesting their neuronal differentiation ability (Figure 3D).

**Transplanted Muse Cells Aid in Pyramidal Tract Reconstruction**

The anterograde tracer, dextran, when injected into the motor cortex labeled the host pyramidal tract. Focusing on the region close to the lacunar infarct lesion, the stump of dextran-labeled pyramidal tract displayed synaptophysin positivity and was detected adjacent to GFP-muse cells, suggesting proximal juxtaposition between neurites of motor cortex neurons and muse cells transplanted at the perilesion site (Figure 4A). On the contrary, when injected into the perilesion site, dextran incorporated into the transplanted muse cells and was transported through the ipsilateral pyramidal tract (Figure 4B1).
to the contralateral side at the level of medulla (Figure 4B2), and then transported through the contralateral pyramidal tract in the dorsal funiculus of spinal cord, specifically recognized at the level of upper cervical spinal cord (C1-2) (Figure 4B3). Because majority of neuritis in the same region was shown to be interrupted in C2 level spinal cord at 8 weeks (Figure 1B), the dextran-positive signals (recognized in Figure 4B3) likely represented newly regenerated nerve fibers. Furthermore, VGluT (glutamatergic neuronal marker)–positive muse cells, which positively stained with dextran and synaptophysin, were observed in the anterior horn of cervical spinal cord (Figure 4B4 and 4B5).

Transplanted Muse Cells Improve Behavioral Score
Corner turn test at 6 and 8 weeks demonstrated significant recovery in the muse-rich group compared with the MSC (*P<0.05) and vehicle groups (*P<0.05; Figure 5A). Cylinder test at 4, 6 (both for MSC and vehicle groups; *P<0.05), and 8 (both at **P<0.01) weeks revealed significant improvement of the neurological performance in the muse-rich group compared with that in the MSC and vehicle groups (Figure 5B).

Treatment with diphtheria toxin, known to selectively ablate human cells in rodent model, deteriorated behavioral recovery, with the functional deficit resembling that of the vehicle group (**P<0.01; Figure 5C).

Safety Assessment of Muse Cell Grafts
Human-specific Alu sequence was detected only in the brain, which was more pronounced at 6 months compared with 2 months post-transplantation of muse-rich cells in stroke animals, but was not found in any organ at 10 months post-transplantation in intact mice (Figure 6A). Tumor formation was not evident in any organ investigated (Figure 6B).

Discussion
The present study used the subacute phase (ie, 2 weeks post-injury) lacunar infarct model to reveal the effectiveness and safety of locally injected human muse cells in severe combined immunodeficiency mice. In addition to FACS-isolated muse cells cultured in serum/xeno-free system that is free from potential cross-species contaminants, we also examined MACS-sorted muse-rich cells under the same culture system.
Our present data demonstrated that both FACS-isolated and MACS-sorted muse cells retained their neuronal differentiation ability and afforded robust and stable therapeutic benefits against stroke.

Clinical trials of neural stem cell transplantation were initially implemented to replace degenerated or dead neuronal cells, but to date only scarce reports support the occurrence of neuronal circuitry reconstruction, with safety concerns of tumorigenic potential. Such adverse event of tumor formation is circumvented by MSCs because these cells do not persist in the host brain after transplantation in stroke animal models. Despite lack of graft persistence, MSCs exert neuroprotection via anti-inflammatory and cytokine secretory effects in laboratory studies but clinically relevant outcomes in patients with stroke treated with MSCs warrant further investigations. Subacute (as in the present study of 2 weeks poststroke) and the chronic (1 month poststroke) both represent delayed stages of stroke that preclude stem cell therapy at this late time point as likely abrogating the acute cell death processes of stroke. Interestingly, delayed transplantation of MSCs still produced robust behavioral recovery despite modest graft survival, suggesting that rather than graft persistence, the bystander effects such as endogenous neurogenesis and neuroplasticity, as seen in preclinical studies and alluded to in a clinical study, seem to largely mediate MSC functional benefits. That delayed MSC transplantation induces a cell replacement mechanism remains elusive. Along the same vein, MSCs may confer limited effects when transplanted at the delayed 2 weeks or 1 month poststroke, but even with this delayed transplantation, MSCs remain resilient in inducing behavioral recovery and it is the graft persistence (albeit cell replacement) that becomes highly restricted. To this end, the effectiveness of MSCs especially in delayed transplantation regimen is largely attributed to the bystander effects and not on cell replacement. Conversely, and henceforth advances the main tenet of our study, the beneficial effects of muse cells entail the cell replacement mechanism even when transplanted at the late phase of stroke. This novel observation of cell replacement at the delayed stage of stroke suggests that muse cells differ from MSCs in that they persist in the host tissue, replenish neuronal cells, and participate in structural regeneration, in particular, neuronal circuitry reconstruction that renders sustainable functional recovery in the stroke brain. However, whether muse cells equally deliver robust bystander effects as seen in MSCs was not examined in this study. Accordingly, MSCs and muse cells may possess unique therapeutic mechanisms in attenuating stroke deficits, indicating perhaps a combination of MSCs and muse cells in the proper proportion may synergistically enhance the functional outcomes of cell therapy.

Eight weeks after transplantation, the majority of engrafted muse cells differentiated spontaneously into NeuN-positive (62.2±2.4% of GFP-positive cells) and MAP2-positive (30.6±3.1%) cells that engrafted close to the lesion site. Muse cells did not disperse widely throughout the brain probably because the damaged site was focal. Furthermore, they differentiated into GST-pi-positive (12.1±1.1%) cells, suggesting that their commitment was not only confined to neuronal cells but also included an oligodendrocyte phenotype. Interestingly, muse cells did not differentiate into astrocyte and microglia, likely because of microenvironmental cues. Damaged cells in the lesion area comprised primarily of neuronal cells and oligodendrocytes, with only minimal proportion of astrocytes, which likely influenced the low differentiation capacity of muse cells toward the astrocytic phenotype.

![Figure 5. Behavioral analysis and loss of function study. The number of animals in each group was 7. Transplantation of muse-rich cells resulted in functional recovery in corner turn (A, 0.5: no deficit, scores higher or lower than 0.5: severe deficit) and cylinder (B, 1: no deficit, 0: severe deficit) test with statistical differences to the mesenchymal stem cell (MSC) and vehicle groups. The functional recovery was ablated by the administration of diptheria toxin (DT; C, 1: no deficit, 0: severe deficit). *P<0.05, **P<0.01, ***P<0.001.](http://stroke.ahajournals.org/)}
Neuron tracing results showed that transplanted muse cells extended their neurites into the contralateral site, crossing at the medulla, and reached at least the upper cervical spinal cord (C1-2). Neurites of muse cells in the spinal cord expressed VGluT and synaptophysin, markers for glutamatergic neurons and presynapses. Transplantation of muse-rich cells significantly improved the neurological performance compared with MSC and vehicle treatments. In addition, human-selective toxin diphtheria toxin abrogated the functional recovery produced by muse-rich cell transplantation, suggesting that successful integration of muse cells into host neural network by connecting to motor neurons likely mediated the behavioral outcome.

Functional recovery may be related to \( \approx 71\% \) purity of the muse cell, which was achieved here via serum/xeno-free system and the clinically accepted MACS set-up.\(^\text{18}\) Of note, animals that received MSC transplantation, which contained a small fraction of muse cells, did not demonstrate significant better recovery compared with vehicle-treated animals, which is consistent with a previous report demonstrating that MSC transplantation was not effective when initiated in the late phase of stroke.\(^\text{5}\) Here, we provide evidence that muse cells, as opposed to a general MSC phenotype, may facilitate improved cell graft survival, integration into the host brain, and differentiation into neuronal cells, which stand as key components for initiating neuronal circuitry reconstruction even in the delayed subacute phase of stroke.

MSCs as donor transplantable cells are nontumorigenic, thereby garnering a solid safety record in the clinic,\(^\text{29,30}\) but the surgical procedure of cell transplantation may generally have potential detrimental effects such as infusion toxicity, infection, and formation of tumor.\(^\text{30}\) We showed here that muse cells were also safe, with no tumor formation detected up to at least 6 months post-transplantation. In lacunar infarcted mice that received MACS-sorted muse-rich cells, human-specific Alu sequence was detected only in the brain, indicating that intracerebral local injections of muse cells did not migrate to peripheral organs. Neither ectopic tissue formation nor unregulated Ki67 proliferation of muse cells was observed in the brain and other organs examined. That intact mice transplanted with MACS-sorted muse-rich cells exhibited no human-specific Alu in organ including the brain even though muse-rich cells were directly injected into the brain, suggests that muse cells engrafted only into appropriate injured site (ie, damaged area), but not when vacant seat (loss of cells) was not provided by the microenvironment. Nevertheless, potential detrimental effects need to be validated in higher mammals such as nonhuman primates in future study.

Transplantation of human muse cells in the subacute phase of lacunar infarcts in severe combined immunodeficiency mice produced favorable neurological recovery, accompanied by grafted muse cells differentiating into neurons and oligodendrocytes, and participating in the reconstruction of pyramidal tract. Combined with the observed safety outcomes in the long-term graft maturation period, the present study advances the use of muse cells for transplant therapy in stroke.

Figure 6. Safety evaluation. Quantitative polymerase chain reaction for human-specific Alu sequence was acquired from lacunar infarcts model 2 and 6 mo after muse-rich group and from intact model 10 mo after muse-rich cell transplantation (A). Human-specific Alu signal was detected only in the brain of lacunar infarcts model 2 and 6 mo after transplantation. Histologically, tumor formation was not recognized in any organ (the brain, lung, kidney, liver, and spleen) at 6 mo after transplantation (B). Scale bar, 1000 µm.
Sources of Funding
This study was supported by a Grant-in-Aid from the New Energy and Industrial Technology Development Organization (NEDO), and Grant-in-Aid from Japan Agency for Medical Research and Development (AMED). Dr Borlongan was supported by National Institutes of Health, National Institute of Neurological Disorders and Stroke 1R01NS071956, 1R01NS090962, and 1R21NS089851, Department of Defense W81XWH-11-1-0634, SanBio Inc., KM Pharmaceuticals, NeuralStem Inc., International Stem Cell Corp., and Karyopharm Inc.

Disclosures
Drs Uchida, Nizuma, and Tominaga of Department of Neurosurgery, Tohoku University Graduate School of Medicine, and Y. Kushida, S. Wakao, and Dr Dezawa of Department of Stem Cell Biology and Histology, Tohoku University Graduate School of Medicine, which are parties to a codevelopment agreement concluded with Clio, Inc. under subsidy from the New Energy and Industrial Technology Development Organization and Japan Agency for Medical Research and Development.

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*Stroke*. 2017;48:428-435; originally published online December 20, 2016; doi: 10.1161/STROKEAHA.116.014950

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/48/2/428

Data Supplement (unedited) at:
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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. 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. 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. 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). 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 tracts).

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 microbeads (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. 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. 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, 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 NaO₄, 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. 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 \(\mu\)g/kg) twice at 24 h interval and were reassessed behaviorally at 1 week 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.
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


