研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
北田容章、出澤真理	間葉系幹細胞・Muse細胞を用いた再生医療	岡野栄之 出澤真理	再生医療叢書 7 神経系	朝倉書店	日本	2013	163-187
出澤真理	Muse細胞の発見と再生医療への応用可能性	田中正躬	幹細胞技術の標準化ー再生医療への期待	日本規格協会	日本	2013	22-41

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Wakao S, Matsuse D, Dezawa M.	Mesenchymal Stem Cells as a Source of Schwann Cells: Their Anticipated Use in Peripheral Nerve Regeneration.	Cells Tissues Organs	—	[Epub ahead of print]	2015
Yamauchi T, Kuroda Y, Morita T, et al	Therapeutic Effects of Human Multilineage-Differentiating Stress Enduring (MUSE) Cell Transplantation into Infarct Brain of Mice.	PLoS One	10(3)	e0116009	2015
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RESEARCH ARTICLE

Therapeutic Effects of Human Multilineage-Differentiating Stress Enduring (MUSE) Cell Transplantation into Infarct Brain of Mice

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Abstract

Objective

Bone marrow stromal cells (BMSCs) are heterogeneous and their therapeutic effect is pleiotropic. Multilineage-differentiating stress enduring (Muse) cells are recently identified to comprise several percentages of BMSCs, being able to differentiate into triploblastic lineages including neuronal cells and act as tissue repair cells. This study was aimed to clarify how Muse and non-Muse cells in BMSCs contribute to functional recovery after ischemic stroke.

Methods

Human BMSCs were separated into stage specific embryonic antigen-3-positive Muse cells and -negative non-Muse cells. Immunodeficient mice were subjected to permanent middle cerebral artery occlusion and received transplantation of vehicle, Muse, non-Muse or BMSCs (2.5×10^4 cells) into the ipsilateral striatum 7 days later.

Results

Motor function recovery in BMSC and non-Muse groups became apparent at 21 days after transplantation, but reached the plateau thereafter. In Muse group, functional recovery was not observed for up to 28 days post-transplantation, but became apparent at 35 days post-transplantation. On immunohistochemistry, only Muse cells were integrated into peri-infarct cortex and differentiate into Tuj-1- and NeuN-expressing cells, while negligible number of BMSCs and non-Muse cells remained in the peri-infarct area at 42 days post-transplantation.

Conclusions

These findings strongly suggest that Muse cells and non-Muse cells may contribute differently to tissue regeneration and functional recovery. Muse cells may be more responsible

meet, ICMJE criteria for authorship. All contributing authors are aware of and agree to the submission of this manuscript.

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for replacement of the lost neurons through their integration into the peri-infarct cortex and spontaneous differentiation into neuronal marker-positive cells. Non-Muse cells do not remain in the host brain and may exhibit trophic effects rather than cell replacement.

Introduction

Cell transplantation therapy has been expected to promote functional recovery in various kinds of central nervous system (CNS) disorders including cerebral infarct. The bone marrow stromal cells (BMSCs) may have the enormous therapeutic potential because they can be harvested from the patients themselves and donors without posing ethical or immunological difficulties [1–3]. Based on recent knowledge, allogeneic BMSC transplantation may also be available [4]. More importantly, they are non-tumorigenic and are already applied to the patients with CNS disorders, thus they are highly feasible [5]. The BMSCs are non-hematopoietic cells and are also known as mesenchymal stromal cells [1,2]. For the decades, numerous numbers of studies have indicated that the transplanted BMSCs enhance motor function recovery after the insults in animal models of various neurological disorders, including cerebral infarct [3,6–9]. They also have the potential to ameliorate cognitive dysfunction under certain conditions in diffuse axonal injury and chronic cerebral ischemia models [10,11]. However, there are many variables that may affect the efficacy of BMSC transplantation in the clinical setting. They include donor cell factors (safety, autologous or allogeneic, ex vivo cell expansion), patient factors (age, stroke type), treatment factors (interval since onset, delivery route, cell dose), and validation factors (neurological assessment, imaging) [1]. More importantly, the mechanisms through which the BMSCs promote functional recovery should be clarified. Thus, these functional recoveries may be based on pleiotropic effects of BMSCs, including inflammation modulation and production of neurotrophic factors, as well as replacement of lost neuronal cells by neuronal differentiation of BMSCs. Such multiple properties may result form heterogeneity of BMSCs [12]. Since the geometry of BMSCs is still obscure, however, the cells responsible for neuronal differentiation are not clarified yet. Nevertheless, if the cells that can be integrated into the damaged CNS tissue and spontaneously differentiate into neuronal cells are identified in BMSCs, those would be ideal for regenerative medicine of CNS disorders, and would be expected to improve the efficiency of currently performed BMSC transplantation [1,2].

Recently, multilineage-differentiating stress enduring (Muse) cells are identified in BMSCs [13]. They correspond to several percentages of total BMSCs, and can be efficiently isolated as cells positive for well-known human embryonic stem (ES) cell marker, stage specific embryonic antigen-3 (SSEA-3). Muse cells can self-renew, express a set of genes associated with pluripotency such as Nanog, Oct3/4 and Sox2, and are able to differentiate into endodermal-, ectodermal-, and mesodermal-lineage cells from a single cells. Under cytokine induction, Muse cells differentiate into neuronal maker positive cells with very high ratio of ~90% [14]. Interestingly, they act as tissue repair cells when transplanted *in vivo*; they migrate toward and home into damaged tissues and spontaneously differentiate into cells compatible with the homed-into tissue in fulminant hepatitis, muscle degeneration and skin injury models [13]. Unlike well-known pluripotent stem cells such as ES cells and induced pluripotent stem (iPS) cells, their telomerase activity is low and do not form teratoma in immunodeficient mice testes [14,15]. In contrast, the remainder of BMSCs, non-Muse cells, does not originally express pluripotency genes, nor do they self-renew, differentiate into triploblastic lineages or function as tissue repair

cells *in vivo* [14,15]. These results strongly suggest that Muse cells may play a major role in the neural differentiation and thus may directly contribute to tissue regeneration of damaged CNS, although they are only several percentage of total BMSCs. In the past decade, most of transplantation experiment of BMSCs into ischemia model have been conducted by a mixture of heterogeneous BMSCs, and analysis based on a certain subpopulation in BMSCs have not been focused yet.

In this study, therefore, the authors separated human BMSCs into Muse and non-Muse cells, and transplanted each of them into focal cerebral ischemia model to analyze their contribution to tissue regeneration and functional recovery. They also compared the effect exerted by Muse and non-Muse cell transplantation with that of regular BMSC transplantation.

Materials and Methods

Cell preparation

Human BMSCs were purchased from Lonza Co. The cells were plated at $5.0\times10^5/75cm^2$ in non-coated flask (EasyFlask 159910; Nunc) in α-MEM (Sigma), 10% fetal bovine serum (FBS; Gibco), and 1% kanamycin (Invitrogen). They were incubated at 37°C and 5%CO_2. The culture medium was replaced 3 times a week. When the cells were grown to confluence, the cells were lifted by 0.25% trypsin and 0.02% EDTA in PBS. The cells were passed 3 times before cell sorting. To obtain Muse cells, human BMSCs were incubated with rat anti-SSEA-3 IgM antibody (1:50; Millipore, Billerica, MA; detected by fluorescein isothiocyanate-conjugated anti-rat IgM, Jackson Immunoresearch, West Grove, PA) in the FACS antibody diluents and sorted by Special Order Research Products FACSAriaII (Becton Dickinson, Franklin Lakes, NJ) as described previously [13,14]. Cells negative for SSEA-3 were collected as non-Muse cells, and cells unsorted were used as BMSCs.

For preparation of green-fluorescent protein (GFP)-labeled cells, BMSCs were introduced with GFP-lentivirus at the efficiency of ~80% as described previously. GFP-expressing BMSCs (GFP-BMSCs) were selected by the expression of GFP by FACS, and GFP-Muse (GFP (+) / SSEA-3 (+)) and non-Muse (GFP (+) / SSEA-3 (-)) were separated by the expression of SSEA-3 and GFP as described by Kuroda et al. (2010) [13].

Mice permanent middle cerebral artery occlusion model

All animal experiments were approved by the Animal Study Ethical Committee of Hokkaido University Graduate School of Medicine. Male 6-week-old severe combined immunodeficiency (SCID) mice (n = 24) were purchased from CLEA Japan, Inc. (Tokyo, Japan). Permanent middle cerebral artery (MCA) occlusion was induced as described previously with minor modifications [16,17]. Briefly, anesthesia was induced with 4.0% isoflurane in N_2O:O_2 (70:30) and maintained with 2.0% isoflurane in N_2O:O_2 (70:30). A 1.0-cm vertical skin incision was made between the right eye and ear, and the temporal muscle was mobilized. Under surgical microscope, a 2.0-mm burr hole was made just on the MCA, using a small dental drill. The dura mater was kept intact, and the right MCA was ligated using 10–0 nylon thread through the dura mater. Core temperature was kept between 36.5 and 37.5°C during and after the procedures. Only animals that circles towards the paretic side were included in this study. Triphenyltetrazolium chloride (TTC; Sigma) staining was performed to quantify infarct volume at 24 hr after the onset of ischemia (n = 4) [18]. Infarct volume was quantified in each animal according to the method described previously [19].

Cell transplantation

Vehicle, Muse cells, non-Muse cells, or BMSCs (n = 5 in each group) were transplanted into the ipsilateral striatum at 7 days after the onset of permanent MCA occlusion [17,20]. The timing of transplantation was determined according to previous data that the BMSC given 7 days after injury led to significantly larger numbers of surviving cells than immediate treatment and significant improvements of gait [21]. In fact, we have confirmed that the BMSCs significantly promote functional recovery after ischemic stroke when directly injected into the ipsilateral striatum at 7 or 28 days after the onset [22–24]. Briefly, the animals were fixed to a stereotactic apparatus under deep anesthesia with 4.0% isoflurane in N_2O:O_2 (70:30), and the cranium was exposed through midline skin incision. A burr hole was made 2 mm right to the bregma, using a small dental drill. A Hamilton syringe was inserted 3 mm into the brain parenchyma from the surface of the dura mater, and 10 μL of cell suspension (2.5×10^4 cells) or 10 μL of vehicle (PBS) were introduced into the striatum during a period of 5 minutes, using an automatic microinjection pump. Cell dose was determined on the basis of previous data; $1\sim2 \times 10^5$ of BMSCs can significantly promote functional recovery after ischemic stroke in the rats whose brain is ~2.0g. The weight of mouse brain is ~0.4g and therefore we transplanted 2.5×10^4 cells in this experiment [22,25,26].

Motor function test

Motor function of the animals was serially assessed before and at 1, 7, 14, 21, 28, 35, 42, and 49 days after the onset of ischemia, using a Rotarod treadmill. This behavioral test was performed in all the Muse cell-, non-Muse cell-, BMSC-, and vehicle-treated mice. The Rotarod was set to the acceleration mode from 4 to 40 rpm for 3 minutes. The maximum time that the animal stayed on the Rotarod was recorded for each performance [27].

Spatial memory test

Using an eight-arm radial maze test, spatial memory was serially examined before and at 7 and 35 days after the onset of ischemia. The maze consisted of a central platform (24 cm in diameter) with eight arms that extended radially. The mice were allowed to visit each arm to eat eight pellets in food cups located near the end of each arms. Each animal was trained once per day to memorize the apparatus. Their performance in each trial was assessed using two parameters: number of correct choices in the initial eight chosen arms, and number of errors (defined as choosing arm that had already been visited). When the animals made seven or eight correct choices and no more than one error in three successive sessions, they were deemed to have memorized the maze. In other words, the animals had acquired spatial memory of the eight-arm radial maze. [28]

Histological analysis

At 42 days after transplantation, the animals were deeply anesthetized with 4.0% isoflurane in N_2O:O_2 (70:30) and transcardially perfused with 4% paraformaldehyde. The brain was removed, immersed in 4% paraformaldehyde for another 2 days, and 10 μm thick cryosections were made. They were then incubated with block solution, and reacted with primary antibody against human mitochondria (mouse IgG, 1:100, Abcam), GFP (chicken IgG, 1:1000; Abcam), Tuj-1 (mouse IgG, 1:200, Sigma), NeuN (mouse IgG, 1:1000, Chemicon,) and GFAP (mouse IgG, 1:300; Sigma). Samples were further incubated with secondary antibodies either to anti mouse IgG or chicken IgG conjugated with Alexa Fluor 488 or 568 (Invitrogen) and counterstained with DAPI (Invitrogen). Then they were examined using a c1si Nikon confocal

microscope system (Nikon, Tokyo, Japan). Using the coronal slice at the level of the striatum (3 slices for each animal), 18 ROIs (800μm x 800μm) were placed in the dorsal neocortex adjacent to the cerebral infarct to count the number of human mitochondria, Tuj-1, NeuN and GFAP-positive cells in each animal.

Statistical analysis

All data were expressed as mean ± SD. Continuous data were compared by one-factor analysis of variance (ANOVA) followed by Bonferroni's test among 4 groups. Values of $P<0.05$ were considered statistically significant. A priori power analysis was employed to determine total sample size, using G*Power Software version 3.1.

Results

Infarct volume of permanent MCA occlusion in SCID mice

All animals could survive after the onset of cerebral ischemia through the experiment. At 24 hr after the onset of ischemia, cerebral infarct was widely distributed in the ipsilateral neocortex. Infarct volume was measured as 22.9 ± 2.9% of the contralateral hemisphere on TTC staining (Fig. 1).

Effect of cell transplantation on motor function recovery

As shown in Fig. 2, all animals exhibited severe neurological deficit during 7 days after the onset of focal cerebral ischemia. There was no significant difference in motor function among 4 experimental groups. At 7 days after ischemia, cell transplantation was performed. The vehicle group did not show any significant improvement of motor function throughout the experiment. BMSC-transplanted animals started to show functional recovery at 21 days after transplantation, compared with vehicle-transplanted animals ($P<0.01$). Stereotactic transplantation of non-Muse cells also significantly promoted functional recovery at 21 days after transplantation ($P<0.01$), but did not show any significant improvement thereafter. Motor function at 28, 35

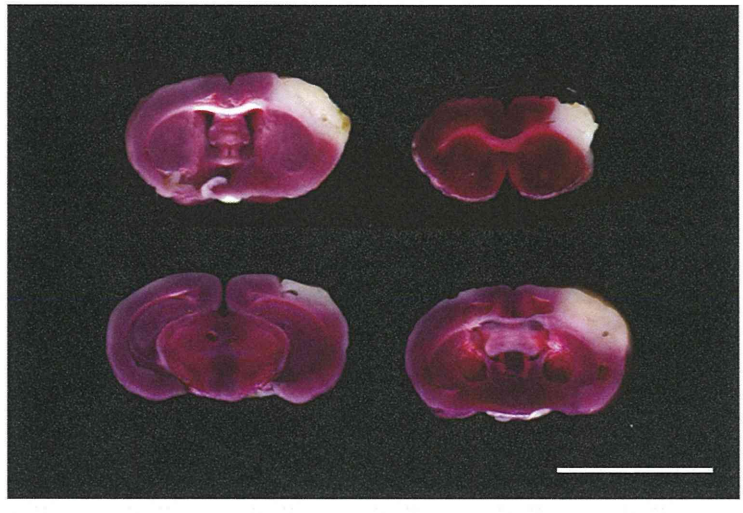

Fig 1. Representative coronal sections of infarct area on triphenyltetrazolium chloride (TTC) staining at 24 hr after permanent middle cerebral artery occlusion. Infarct volume was measured as 22.9 ± 2.9% of the contralateral hemisphere. Scale bar = 5 mm.

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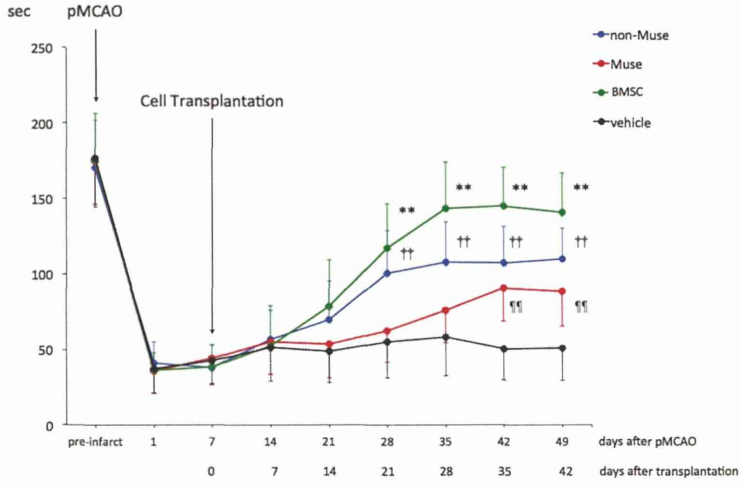

Fig 2. Rotarod treadmill performance. Line graph shows the temporal profile of functional recovery in vehicle-, BMSC-, non-Muse cell-, and Muse cell-treated mice subjected to permanent middle cerebral artery occlusion (pMCAO). **, ††, ¶¶ P<0.01 vs. vehicle-treated mice. Sec indicates seconds.

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and 42 days post-transplantation was significantly better in BMSC group than in non-Muse cell group (P<0.05). In Muse cell group, any therapeutic effect was not observed for up to 28 days after transplantation. However, significant improvement became apparent after 35 days post-transplantation, compared with vehicle group (P<0.01), which was later than BMSC and non-Muse cell groups. Motor function at 35 and 42 days post-transplantation was significantly better in BMSC group than in Muse cell group (P<0.05). There were no significant differences in motor function between Muse and non-Muse groups at 35 and 42 days post-transplantation.

Effect of cell transplantation on spatial memory

The mice subjected to permanent MCA occlusion showed a decrease in the number of correct choices and an increase in the number of errors at 7 days after the onset of ischemia (Fig. 3).

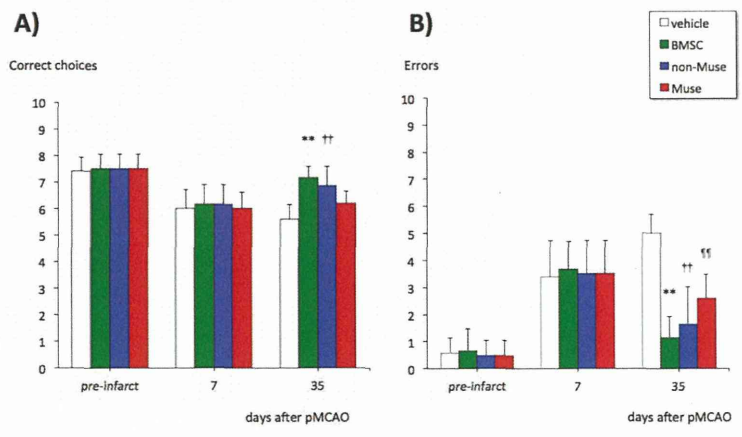

Fig 3. Effects of cell therapy with vehicle, BMSCs, non-Muse cells, and Muse cells on memory impairment at 7 and 35 days after permanent middle cerebral artery occlusion (pMCAO). Correct choices (A) and errors (B) in the eight-arm radial maze task. **, ††, ¶¶ P<0.01 vs. vehicle-treated mice.

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Stereotactic transplantation of BMSCs and non-Muse cells, but not of Muse cells, significantly increased the number of correct choices at 4 weeks post-transplantation, compared with the vehicle-transplanted animals. However, stereotactic transplantation of BMSCs, non-Muse cells, and Muse cells significantly decreased the number of errors at the same timing at the same timing (Fig. 3).

Detection of transplanted cells in the host brain

Histological analysis was performed at 42 days post-transplantation, *i.e.*, at 49 day post-ischemia. Transplanted human BMSCs, Muse and non-Muse cells were detected by anti-human mitochondria. BMSC and non-Muse cell groups did not show any efficient integration of the transplanted cells (Fig. 4). Green fluorescent signals were seen in the peri-infarct area; the area that is located adjacent to the lost lesion. However, these were in most cases autofluorescence of phagocytic cells and were not by the immunoreactions to human mitochondria, thus the number of integrated human BMSCs or non-Muse cells was considered negligible (Fig. 4). In sharp contrast, numerous numbers of integrated human cells were widely distributed in the peri-infarct area of the ipsilateral hemisphere in Muse cell group. The number of human mitochondria-positive cells was 128.3 ± 41/mm^2, which was significantly higher number than that in BMSC- and non-Muse cell groups, 8.5 ± 5.0/mm^2 and 2.5 ± 0.8/mm^2, respectively ($P<0.01$, Fig. 4).

Fluorescence immunostaining of Muse cell group samples demonstrated that the GFP-positive transplanted human Muse cells distributed in the peri-infarct area expressed neuronal markers, Tuj-1 (45.3 ± 13.9% of total GFP+ cells, Fig. 5A and B, Fig. 6) and NeuN (20.5 ± 8.7% of total GFP+ cells Fig. 5C, Fig. 6). However, only a small number of human mitochondria-positive Muse cells were positive for astrocyte/neural stem cell marker, GFAP (1.4 ± 1.2%; Fig. 5D, Fig. 6), suggesting that majority of integrated Muse cells spontaneously differentiated into neuronal marker-positive cells.

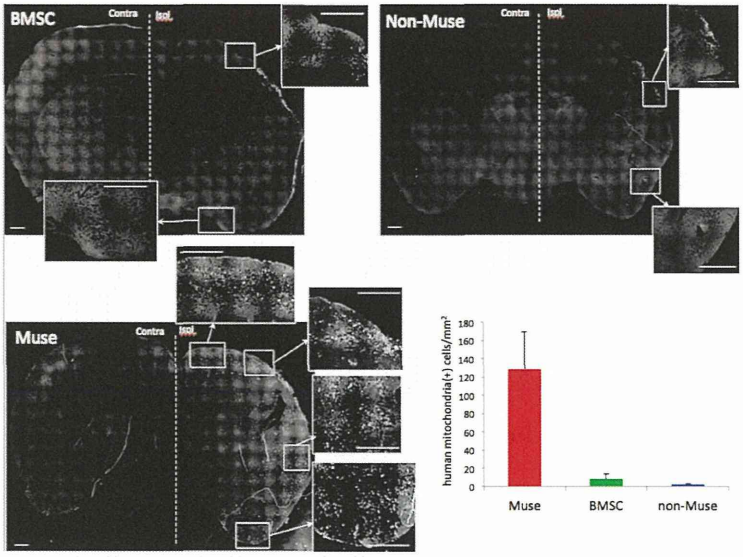

Fig 4. Low-power photomicrographs of fluorescence immunohistochemistry using anti-human mitochondria antibody in BMSC-, non-Muse cell-, and Muse cell-treated mice at 42 days after transplantation. A large number of human mitochondria-positive cells are engrafted in the peri-infarct area in Muse cell group. Graph shows number of human mitochondria-positive cells/mm^2 in ipsilateral cortex of each group. Scale bars = 500 μm.

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