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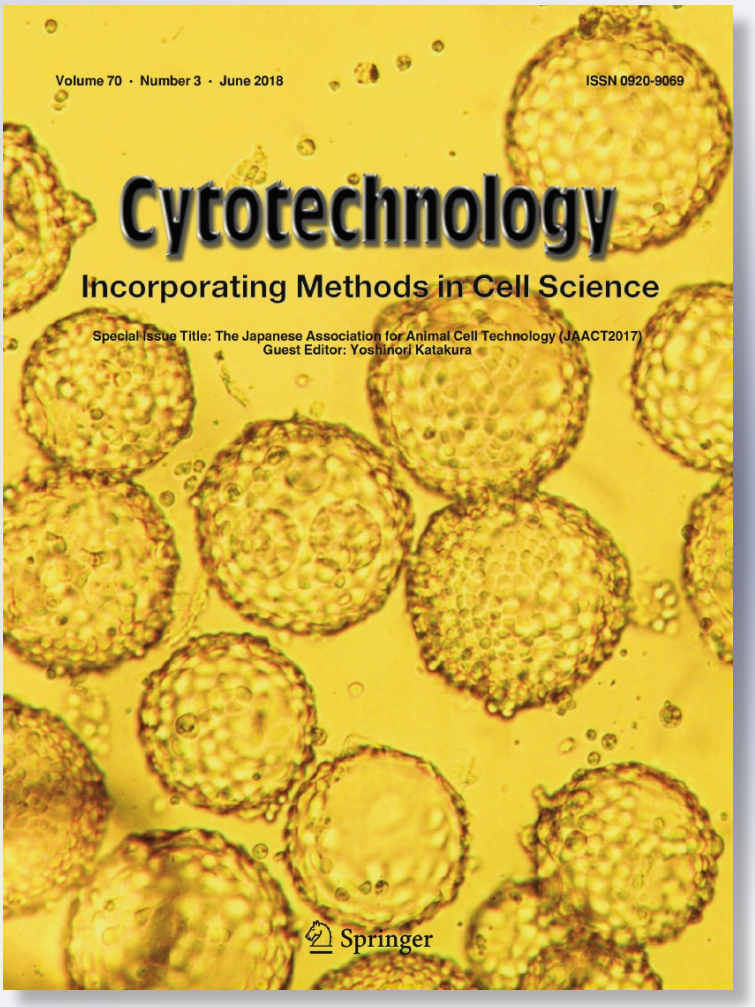

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ORIGINAL ARTICLE

Functional recovery by application of human dedifferentiated fat cells on cerebral infarction mice model

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Abstract Elderly people whose daily activities have declined due to a cerebrovascular disorder may suffer from dysphagia and may find oral hygiene difficult. Therefore, it is important to establish an effective therapy for the underlying cerebrovascular disorder. Dedifferentiated fat cells (DFAT) were obtained from mature adipocytes isolated from human buccal adipose pads in a ceiling culture. DFAT expressed the neural markers Nestin and SOX2. Flow cytometric analysis revealed that the cells had properties similar to mesenchymal stem cells. Although the transplantation of DFAT did not change the infarction area and volume ratios in a murine cerebral infarction model, functional recovery was observed in behavioral tests. Furthermore, DFAT administered to mice were later detected in cerebral infarctions. It therefore appears that transplanted DFAT affect the brain after infarction and contribute to the promotion of functional recovery. This finding may provide new cell replacement therapy options for treating disorders of the central nervous system.

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Keywords Cerebrovascular disorder · Transplantation · Adipose stem cell · Cerebral infarction · Neural differentiation · Nestin

Introduction

Elderly people whose daily activities have declined due to a cerebrovascular disorder may suffer from dysphagia and may find oral hygiene difficult. This may lead to an increase in the occurrence of aspiration pneumonia and mortality (Umarova et al. 2007; Rochester and Mohsenin 2002). Therefore, it is important to establish an effective therapy for the underlying cerebrovascular disorder. Cardiovascular regeneration therapy using stem cells for cerebral infarction has gained attention in recent years. Because many patients with cerebral infarction are elderly with other concurrent cardiovascular conditions such as high blood pressure and abnormal heart rhythm, invasive sampling procedures to harvest cells for transplantation from such patients should be avoided as much as possible.

Bone marrow stromal cells (BMSC) are mesenchymal stem cells (MSC) that have been reported to differentiate not only into ectoderm-derived bone, cartilage, and fat cells but also into endoderm-derived nerve cells. The effectiveness of central nervous system regeneration using BMSC has been noted in animal experiments (Kurozumi et al. 2004, 2005) and

clinical research (Bang et al. 2005). However, lumbar puncture, which is required to extract BMSC, is sometimes invasive; thus, a non-invasive method may be recommended. In contrast, adipose stem cells (ASC), which are MSC-like BMSC established from adipose tissue, are good candidates for clinical use as transplanted cells due to minimal invasiveness. The utility of the transplantation of ASC for neuronal regeneration in the central nervous system has been reported in animal studies (Gutiérrez-Fernández et al. 2013b; Ikegame et al. 2011), and clinical research is currently underway (Gutiérrez-Fernández et al. 2013a). However, both BMSC and ASC derived from the bone marrow or fatty tissue exhibit non-uniform cell populations. With regard to safety in clinical applications, it is desirable to use high-purity cell populations (uniform cells) as cells for transplantation.

Alternatively, dedifferentiated fat cells (DFAT), which can be obtained from mature fat tissue by ceiling culture method, show uniform cell population (Ohta et al. 2008). Ohta et al. also noted that neuronal markers are expressed in primary undifferentiated DFAT suggesting that these cells essentially have neural traits. Subsequently, the transplantation of DFAT has been reported to show the recovery of motor function of the hind limbs in a rat spinal cord injury model, indicating the efficacy of the transplantation of DFAT in the central nervous system. However, the effects in a cerebral infarction model have not yet been reported. We hypothesized that the transplantation of DFAT in an animal model of cerebral infarction has a neural regenerative effect on cerebral infarction. Thus, the purpose of this study was to evaluate the effect of DFAT transplantation on cerebral infarction both morphologically and functionally in a reproducible and simple model of cerebral infarction in mice.

Materials and methods

Animal studies

We used 5-week-old male C.B-17/Icr-+/+Jcl mice (CLEA Japan, Inc., Tokyo, Japan). The mean weight is 27.5 g. Treatment of all animals was conducted in compliance with the guidelines of Osaka Dental University Animal Experiment Committee and "Guidelines on Animal Protection, Welfare and Use" of the Biological Science Research Committee. Animals were provided with food and water ad libitum and were maintained in an environment with a 12-h light–dark cycle. We made every effort to minimize their pain and the number of animals used.

Isolation and ceiling culture of DFAT

We collected human buccal adipose pads from a woman (27 years old) who had undergone oral maxillofacial surgery at Osaka Dental University Hospital. All research procedures were approved by the Medical Ethics Committee of Osaka Dental University (approval number 110,828). We isolated DFAT using the ceiling cultivation method described by Kishimoto et al. (2014). Briefly, approximately 3 g of thinly cut buccal adipose pads were processed in 0.1% (w/v) collagenase solution (collagenase type I; Wako Pure Chemical, Osaka, Japan) at 37 °C for 1 h. The cell suspension was then filtered through 150- and 250-μm nylon meshes to eliminate unwanted cells and tissue. After filtration, the suspension was centrifuged at 1000 rpm for 4 min and the floating layer of oil droplets containing adipose cells was collected. Isolated adipocytes were seeded into 25-cm^2 flasks (SUMILON; Sumitomo Bakelite Co. Ltd., Tokyo, Japan), fully filled with a medium that contained an antibiotic–antimycotic (10,000 U/ml penicillin, 10,000 μg/ml streptomycin, and 25 μg/ml amphotericin B; Nacalai Tesque, Kyoto, Japan) and 20% v/v fetal bovine serum (FBS; lot number AYD62671; HyClone Laboratories Inc., Logan, UT, USA), and then added to Dulbecco's modified Eagle's medium (DMEM; control condition medium; Nacalai Tesque). Flasks were placed with the culture surface facing upward, and lipid droplets including floating adipose cells were incubated at 37 °C in 5% CO_2 to allow for the adhesion of cells to the inner surface of the flask ceiling. After 7 days, the medium was removed and the flask was turned over. Thereafter, normal cell culture was conducted. The medium was replaced every 4 days (Fig. 1). When the cells reached confluence, they were removed with 0.25% trypsin/1 mM EDTA (Thermo Scientific, Rochester, NY, USA) and placed into 75-cm^2 flasks (SUMILON; Sumitomo Bakelite Co. Ltd.) (passage 1). Then, for immunohistochemical staining, DFAT were placed in 12-well plates (AGC Techno Glass Co. Ltd., Tokyo, Japan). After culturing in control medium for 12 days, the

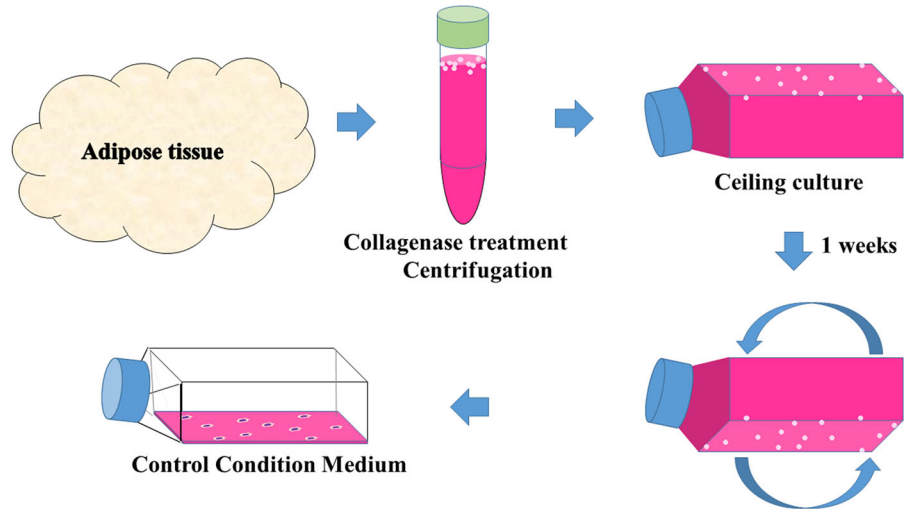

Fig. 1 Isolation and celling culture of human DFAT from human buccal adipose pads. In the ceiling cultivation method, the cells reached confluence after about 1 week. Then the medium was removed and the flask was turned over, normal cell culture was performed

cells were verified for confluence. Cultured DFAT were fixed in 4% paraformaldehyde/phosphate-buffered saline (pH 7.4). Immunohistochemical staining was performed with anti-Nestin antibodies (1:200; Merck Millipore, Darmstadt, Germany), anti-SOX2 antibodies (1:100; Merck Millipore), and CD45 antibody (R&D Systems, Minneapolis, MN, USA).

Flow cytometric analysis

Third-passage DFAT were analyzed for cell surface antigens. The number of cells per assay was 3.5×10^5. A human mesenchymal stem cell marker antibody panel (R&D Systems) was used for the primary antibodies. The antibodies employed were STRO-1, CD19, CD44, CD45, CD90, CD105, CD105, CD146, and CD166. Reaction with primary antibodies occurred at room temperature for 30 min, after which the cells were washed. Alexa Fluor 488-conjugated IgG and IgM antibodies were used as secondary antibodies and the cells were incubated at room temperature in the dark for 30 min. ABD LSR Fortesssa™ X-20 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was employed for flow cytometric analysis. Data analysis was performed with a BD FACStation (Tree Star, Ashland, OR, USA).

Differentiation of DFAT into neural cells

Based on previous reports (Nakagomi et al. 2009a, b; Reynolds and Weiss 1992; Nakata et al. 2017), we conducted differentiation of nerve cells guided by DFAT. DFAT (third passage) were placed in 60-mm-diameter, poly-D-lysine-coated dishes (Thermo Scientific) and cultured in DMEM/F12 (Invitrogen, Carlsbad, CA, USA) with epidermal growth factor (EGF; 20 ng/ml; Peprotech, Rocky Hill, NJ, USA) and fibroblast growth factor-basic (FGF; 20 ng/ml; Peprotech) for 3 weeks. Formed neurosphere-like cell clusters were placed on top of poly-L-lysine-coated (0.05%) glass coverslips (Matsunami Glass Ind., Ltd., Osaka, Japan). The glass coverslips with neurosphere-like cell clusters were placed in 35-mm-diameter dishes (Thermo Scientific) and incubated in neurobasal medium (Neural Condition Medium; Invitrogen) containing retinoic acid (0.06 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) and B-27 supplement (Invitrogen) for 1 week.

Next, clusters of DFAT were subjected to antibodies against TuJ1 (1:1000; Stemcell Technologies, Vancouver, BC, Canada) for immunohistochemical staining. The Alexa Fluor 488-conjugated secondary antibodies (1:500; Molecular Probes, Eugene, OR, USA) stained green. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; 1:1000; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). After staining, cells were observed with a fluorescence microscope (Olympus DP71; Olympus Inc., Tokyo, Japan).

Induction of focal cerebral ischemia and cell transplantation

Permanent cerebral ischemia was induced by electrosurgical occlusion of the left middle cerebral artery of male mice as previously reported (Taguchi et al. 2010). Briefly, while the mice were inhaling sevoflurane, the left middle cerebral artery (MCA) was isolated and a cut was made immediately before MCA crossed the olfactory tube (distal M1 part). The resulting area of cerebral infarction in mice is highly reproducible and has been shown to be limited to the ipsilateral cerebral cortex (Nakagomi et al. 2009a, b; Taguchi et al. 2004, 2007). Two days after inducing cerebral ischemia, 30 µl of phosphate-buffered saline (PBS) (n = 6) or 30 µl of PBS containing 5×10^5 DFAT (after third passage) (n = 10) was injected intravenously into the tail vein. The sham control group contained mice (n = 4) that were subjected to intravenous administration of 30 µL PBS 2 days after drilling the hole in the skull. These mice did not develop cerebral infarction. To assess functional behavior, we conducted behavioral tests for 4 weeks.

Four weeks after cerebral infarction, mice were euthanized to remove their brain tissue and the tissue was fixed in 4% paraformaldehyde/PBS. Images of the whole brain from the parietal side were taken with an Olympus szx12 microscope (Olympus Inc.). The image analysis software Picworks7c (Grambel Precision Inc., Tokyo, Japan) was used. Brain surface areas were measured using The National Institutes of Health ImageJ software (Version 1.51). The infarct side intact cortical surface area was the size of the left cerebral cortex excluding the cerebral infarction site, and the ratio of the infarct side intact cortical surface area to the contralateral cortical (non-infarct side) surface area was calculated. Following immersion and enrichment in sucrose for 3 days, brain tissues were sectioned into 20-µm-thick specimens using a cryostat. Images of these specimens were taken with a BZ-X710 microscope (Keyence Corp., Osaka, Japan). The image analysis software was BX-X Analyzer (Keyence Corp.). Tissue area was measured using ImageJ software. The infarct side intact cortical volume represented the quantity of the left cerebral cortex excluding the cerebral infarction site, and the ratio of the infarct side intact cortical volume to the contralateral cortical (non-infarct side) volume was calculated.

Behavioral tests

Water maze learning (WM) was used to assess motivation. The details of this test are presented below. We used a shallow cylindrical pool (internal diameter 95 cm and height 30 cm) that was fixed horizontally on an installation base of height 35 cm. The platform was a disk of 10 cm in diameter and 1 cm in thickness that was attached to a pedestal, with a total height of 21 cm. The platform surface was placed at the center of any of the four fan-shaped quadrants into which the pool was divided. The shortest distance between the platform edge and the inner wall of the pool was 15 cm. The water level on the surface of the platform was approximately 5 mm. Water was made opaque by the addition of titanium dioxide, and the temperature was maintained at 24 °C ± 1 °C. Each mouse was subjected to five trials per day for 5 consecutive days. In each trial, mice were introduced to a random quadrant where the platform was not located. The mouse was placed with its head turned toward the wall and allowed to swim toward the platform. The time until both forelimbs contacted the platform was measured. The mouse was held for 10 s after reaching the platform. If the mouse did not reach the platform within 60 s after introduction to the pool, the researcher placed it onto the platform and held it there for 10 s. In such cases, escape latency was recorded as 60 s. The interval between trials was 30 s. On the day following the last day of acquisition training, a trial test was conducted by releasing the mouse into the pool from which the platform was removed.

The levels of sensation, perception, and pain susceptibility were evaluated using the hot plate test (HP). An experimental apparatus for mouse hot plate testing (MK-350B: Muromachi Kikai Co., Ltd. Tokyo, Japan) was used. A cylinder made of transparent acrylic (25 cm high) surrounded the hot plate to prevent the mouse from escaping. The surface temperature of the hot plate was adjusted in 2 °C intervals in the range of 50–58 °C, to set five temperature conditions. All mice were subjected to each temperature condition, and measurements of their reactions were made and recorded in ascending order from low- to high-temperature conditions. The interval between trials was 10 min. In each trial, the mouse was exposed to the hot plate surface and the time until it tried to jump away from this surface was noted.

Measurements were terminated at 20 s, and in the event a reaction did not occur, the time was recorded as 20 s. Motor function was evaluated using the wire hang test (WH). A wire plate in which metal wires of 3 mm thickness were arranged in a grid at 1-cm intervals was used. This plate was mounted on the ceiling of a rectangular glass tank (walls of 30 cm in height). Each mouse was subjected twice to wire hanging for 1 min. In each trial, the mouse was held horizontally on the wire plate for approximately 10 s. After confirming that the limbs of the mouse were gripping the wires, the wire plate was inverted 180° and attached to the ceiling of the tank. The time until the mouse dropped to the floor was measured. The measurement was terminated at 180 s and, in the event that the mouse had not dropped to the floor, this was recorded as 180 s.

Antidepressant emotions were evaluated using the forced swimming test (FS). An experimental animal activity monitoring system (Supermex; Muromachi Kikai Co. Ltd.) and control software (CompACT FSS2 ver2; Muromachi Kikai Co. Ltd.) were employed. The apparatus was a soundproofed, rectangular chamber [40 × 60 × 40 cm (width × length × height)] fitted with an infrared sensor (Supermex sensor; Muromachi Kikai Co. Ltd.) in the middle of the ceiling. Directly beneath the infrared sensor inside the chamber, a plastic cylindrical tank (diameter 18 cm, height 29 cm) was filled to a depth of 15 cm with water (24 ± 1 °C). The chamber interior was equipped with white LED and, when illuminated, the amount of light was 150 lx in tank water. In addition, a small ventilation fan was attached to the rear of the chamber to provide ventilation and mask noise. Sound pressure was approximately 45 dB(C) at the floor in the center of the chamber. Each mouse was subjected to 6 min of forced swimming and individually released into the cylindrical water tank. All reactions arising from forced swimming were detected every second and comparatively quantified, and the total was considered as the amount of activity. Further, there was visual assessment every second, and if the movements were less than the reference value of "there was no reaction other than floating on the water," it was defined as an immobile state. The total time spent immobile was also calculated. Despair and depression-like behaviors were assessed using the tail suspension test (TS). An experimental animal activity monitoring system (Supermex) and control software CompACT FSS2 ver2 (Muromachi Kikai Co. Ltd.) were employed. The apparatus was a soundproofed, rectangular chamber [40 × 60 × 40 cm (width × length × height)] fitted with an infrared sensor (Supermex sensor) in the middle of the ceiling. Directly beneath the infrared sensor in the chamber, a plastic rod for hanging the mouse was horizontally fixed at a height of 30 cm from the floor. During the experiment, a compact extractor fan mounted on the chamber wall constantly provided ventilation, and the sound was used as masking noise. Sound pressure was approximately 45 dB(C) at the floor in the center of the chamber. Each mouse was hung for 6 min from the abovementioned plastic rod with a metal hook attached to perforated plastic tape wrapped 2 cm from the tip of the tail. The distance between the head of the mouse and the floor of the soundproof chamber was approximately 10 cm. The tail suspension procedure detected the subject's struggle reaction every second and activity intensity was quantified each minute.

Tracking of transplanted cells

A Vybrant Multicolor Cell-Labeling Kit (Dil) (Molecular Probes Inc., OR, USA) was used to label 5×10^5 DFAT (after the third passage). Two days after inducing cerebral infarction, labeled cells were administered intravenously to mice (n = 4). On the following day, mice were deeply anesthetized with pentobarbital (50 mg/kg). Then, after euthanasia by cervical dislocation, the spleen, lungs, liver, and brain were removed. Each organ was fixed in 4% paraformaldehyde/PBS. Specimens of 12 μm thickness were made using a cryostat. Images were taken with a fluorescence microscope (Olympus DP12; Olympus).

Statistical analysis

The results are shown as mean ± SEM. To analyze brain area, volume, and ratios, SPSS Ver12.0 for Windows (SPSS Inc., Chicago, IL, USA) was used and the Kruskal–Wallis test was employed for statistical analysis. For behavioral tests, analysis of variance was performed and multiple comparisons were statistically analyzed by Fisher's least significant difference test. If asymptotic significance probability $P < 0.05$ was met, we considered the difference to be statistically significant.

Results

Characteristics of DFAT

DFAT cultured in control condition medium resembled fibroblastic cells at the whole-cell level. Expression of Nestin and SOX2, but not CD45, was observed in them using immunohistochemistry. Flow cytometry indicated that, as cell surface markers, STRO-1, CD44, CD90, CD105, and CD146 were expressed, but CD19, CD45, and CD106 were not (Fig. 2).

DFAT formed clumps when cultivated as suspended culture in Petri dishes with neural condition medium. When changing to the neural condition medium, the clumps grew for 4–5 days, and then did not subsequently show any changes in size. Culturing clumps on the surface of poly-L-lysine-coated (0.05%) glass coverslips in neural condition medium culminated in differentiation into dendritic-like cells. Immunohistochemical staining resulted in TuJ1 expression (Fig. 3).

Utility of the transplantation of DFAT into mice with cerebral infarction

Nine out of 10 mice transplanted with DFAT survived for 4 weeks. A total of eight mice were analyzed after the exclusion of one mouse in which cerebral infarction had not been successfully induced from among the group administered cells. Results from behavioral evaluations such as WH, WM, and TS showed no difference between mice in the cerebral infarction group transplanted with DFAT compared with the non-transplant group. HP of 56 °C indicated a difference between mice in the cerebral infarction group transplanted with DFAT and the PBS-administered group. In addition, FS at 1 min exhibited a difference between mice in the cerebral infarction group transplanted with DFAT and those in the PBS-administered group (Fig. 4). The infarct side intact cortical surface area (Fig. 5a) and volume (Fig. 5d) and ratios (Fig. 5c, f) were not significantly different between the DFAT-transplant and PBS-administered groups. On the other hand, there was a significant difference between the DFAT-transplant group and the sham

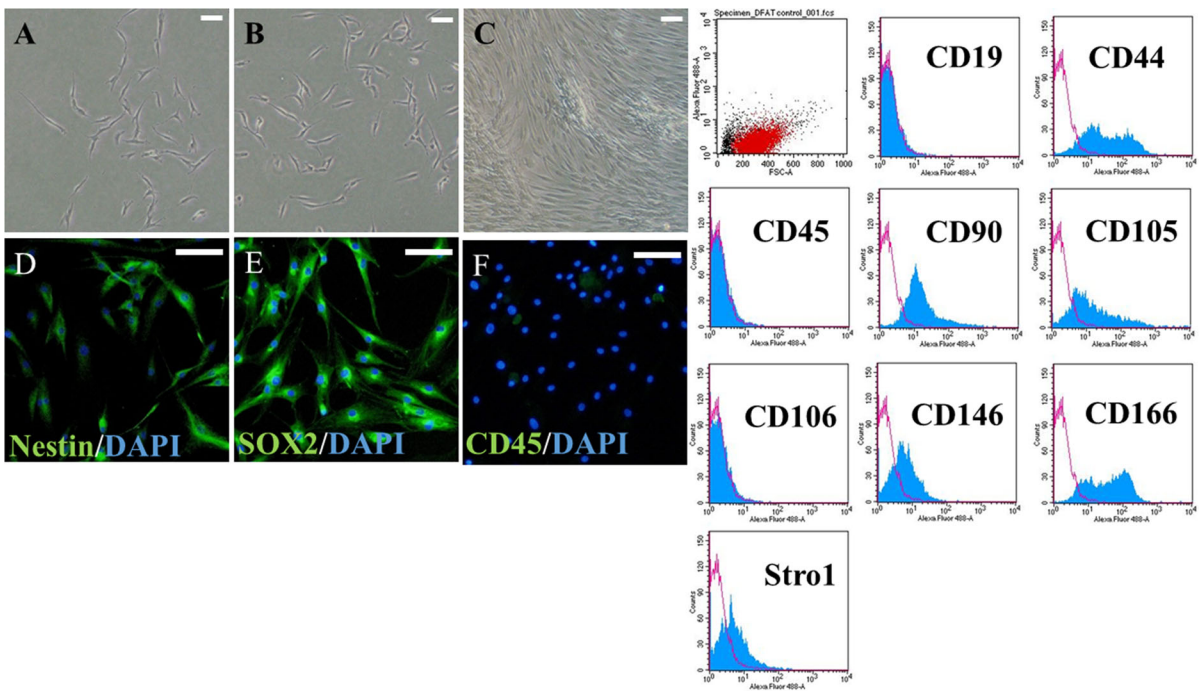

Fig. 2 DFAT characteristics. DFAT culture condition after 5 days (**a**), 9 days (**b**), and 13 days (**c**). Immunohistochemical staining of DFAT (**d**–**f**). Expression of Nestin (**d**) and SOX2 (**e**) appear green, but CD45 is not expressed (**f**). The nucleus is stained blue. Purple open histograms represent antibody isotype controls, and blue closed histograms represent a sample stained with specific antibodies. *Scale bar* = 100 μm (**a**–**c**); 50 μm (**d**–**f**)

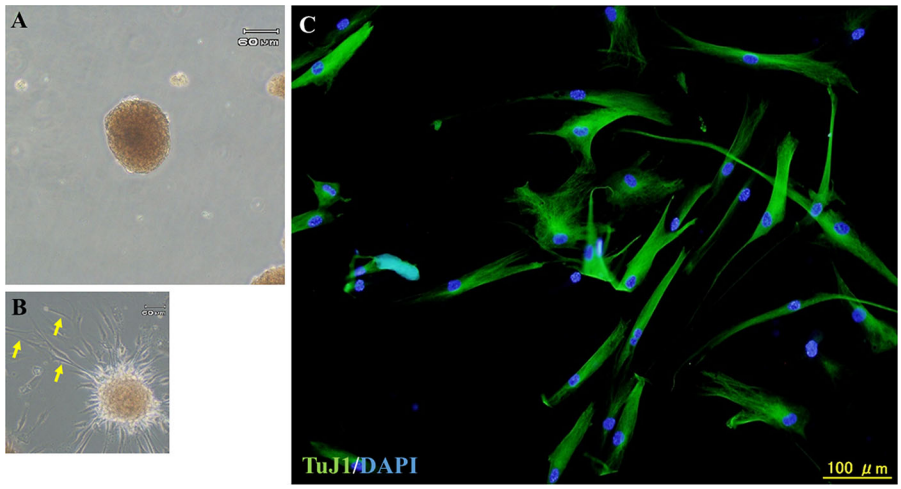

Fig. 3 Neural differentiation of DFAT. Suspended cultures of DFAT form clumps (**a**). When grown on a cell culture adhesion plate, dendritic-like protrusions (yellow arrow) are observed (**b**). Immunohistochemical staining of the dendritic-like protrusions yields TuJ1 expression (**c**)

group, and between the PBS-administered group and the sham group (Fig. 5a, c, d, f). Labeled DFAT was observed in the brain, spleen, liver, and lung (Fig. 5g–j).

In HP, at a high temperature of 56 °C, mice responded more sensitively in the cerebral infarction group transplanted with DFAT than those in the cerebral infarction PBS-administered group. In FS, mice in the cerebral infarction group transplanted with DFAT exhibited shorter immobility time than those in the cerebral infarction PBS-administered group.

Discussion

In this study, we analyzed cells acquired using the ceiling culture method by flow cytometry. The results from analysis of surface antigens of DFAT were consistent with those reported previously (Matsumoto et al. 2008; Patricia et al. 2002). Furthermore, CD105, CD73, and CD90 were expressed in MSC; CD45, CD34, CD14, CD11b, CD79alpha, or CD19 were not expressed in MSC (Dominici et al. 2006). DFAT used in this research expressed CD90 and CD105, but not CD19 or CD45. Thus, it appears that DFAT have similar characteristics to MSC.

DFAT cultured in control condition medium expressed Nestin and SOX2, both are neural stem cell markers. Ohta et al. (2008) have reported that Nestin and GFAP, an astrocyte marker, are expressed even in DFAT that do not show the differentiation. Poloni et al. (2015) also have shown that DFAT forming neurosphere-like cell clusters differentiate into neural cells expressing Tuj1, NSE, MAP2, and GFAP. We found in this study that DFAT-derived neurosphere-like cell clusters culturing in neurobasal medium differentiated into neural cells forming dendrite-like spines. In addition, these cells were shown to express Tuj1, a marker of neural cells. Therefore, it appears that our DFAT underwent neural differentiation in vitro.

We previously conducted a research on bone tissue regeneration using DFAT, in which DFAT have expressed osteoblast markers such as alkaline phosphatase (ALP) osteocalcin (OCN) and calcium similarly to BMSC (Sakamoto et al. 2015). Expression of osteoblast markers (ALP, OCN, and calcium) by DFAT also have been shown in another report (Kishimoto et al. 2014). These studies have suggested that DFAT would have multi-potential to differentiate into cells of various tissues and would be useful for several tissue regenerations.

Unfortunately, the current study showed no significant differences in the infarct side intact cortex area or volume between DFAT and the PBS-administered group of murine cerebral infarction model. Ikegame et al. (2011) intravenously administered MSC sooner after MCA reperfusion using a transient cerebral infarction mouse model, and mice receiving MSC showed a reduction in infarction volume. On the other

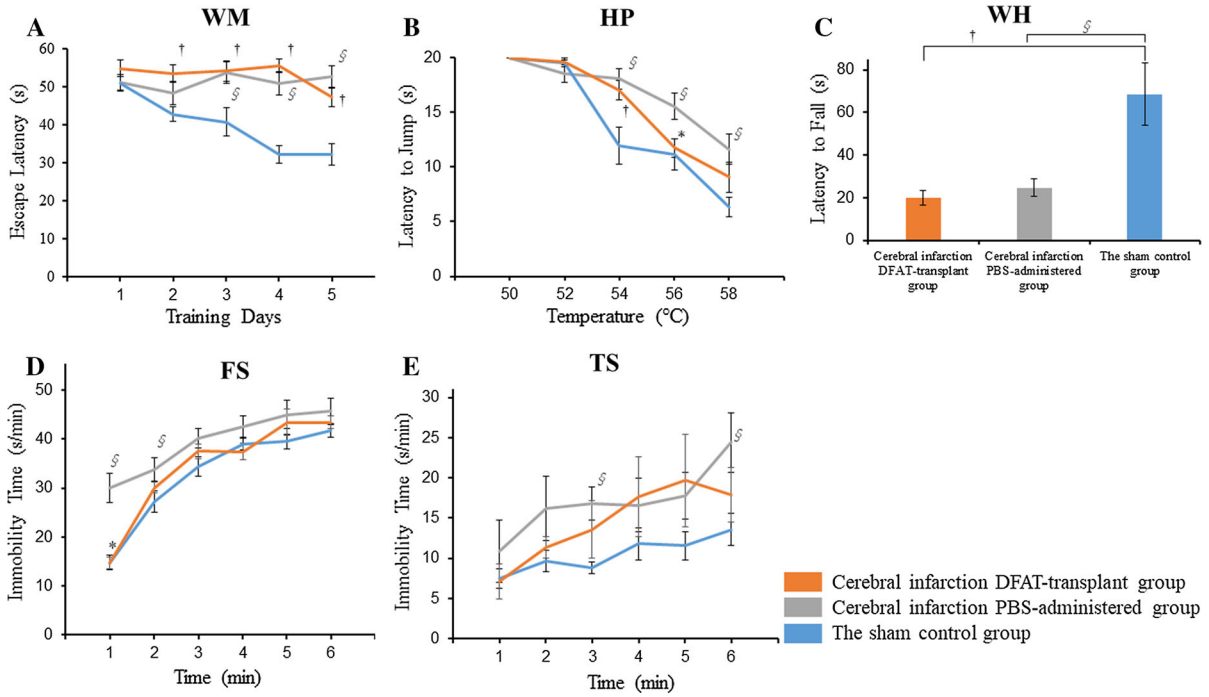

Fig. 4 Results from behavioral tests. **a** In the water maze test (WM), there was no significant difference between the cerebral infarction DFAT-transplant and cerebral infarction PBS-administered groups. **b** In the hot plate test (HP) at 56 °C, there was a significant difference between the cerebral infarction DFAT-transplant and the cerebral infarction PBS-administered groups. **c** In the wire hang test (WH), there was no significant difference between the cerebral infarction DFAT-transplant and the cerebral infarction PBS-administered groups. **d** In the forced swimming test (FS) at 1 min, there was a significant difference between the cerebral infarction DFAT-transplant and the cerebral infarction PBS-administered groups. **e** In the tail suspension test (TS), there was no significant difference between the cerebral infarction DFAT-transplant and the cerebral infarction PBS-administered groups. ($^{*}P < 0.05$ comparison between the cerebral infarction DFAT-transplant group and the cerebral infarction PBS-administered group. $^{\dagger}P < 0.05$ comparison between the cerebral infarction DFAT-transplant group and the sham control group. $^{\S}P < 0.05$ comparison between the cerebral infarction PBS-administered group and the sham control group)

hand, Gutiérrez-Fernández et al. reported that functional recovery was observed after intravenous administration of MSC to a permanent infarct rat model. However, no decrease in the cerebral infarction volume was observed. Although no detailed mechanism was mentioned, it was speculated that the fact that the MCA reperfusion could not be performed in the permanent infarction model was the reason why a reduction in the cerebral infarction volume was not observed (Gutiérrez-Fernández et al. 2013b). In this study, we also used a permanent infarction model so that reperfusion was not necessary. However, this may have led to the absence of a decrease in the cerebral infarct surface area and volume. Because mice in the sham group did not develop cerebral infarction, we speculate that the infarct side intact cortical surface area, volume, and ratio in the sham group were significantly larger than the PBS-administered or DFAT-transplant groups.

Behavioral tests showed significant differences in HP and FS. It is surmised that cell transplantation improved sensory, perceptual, pain sensitivity, and depressant behavior. In contrast, no significant differences were observed for TS, WM, and WH, which can evaluate mainly learning and motor function. These results suggest that the DFAT transplantation by the current method (intravenous injection) has limited effects of functional recovery after cerebral infarction. It was reported that the expressions of neurogenesis, synaptogenesis, angiogenesis, and oligodendrogenesis markers such as NF, Olig-2, SYP, and VEGF were significantly augmented in the brain sections harvested after MSC administration (Gutiérrez-Fernández et al. 2013b). Neurogenesis, synaptogenesis,

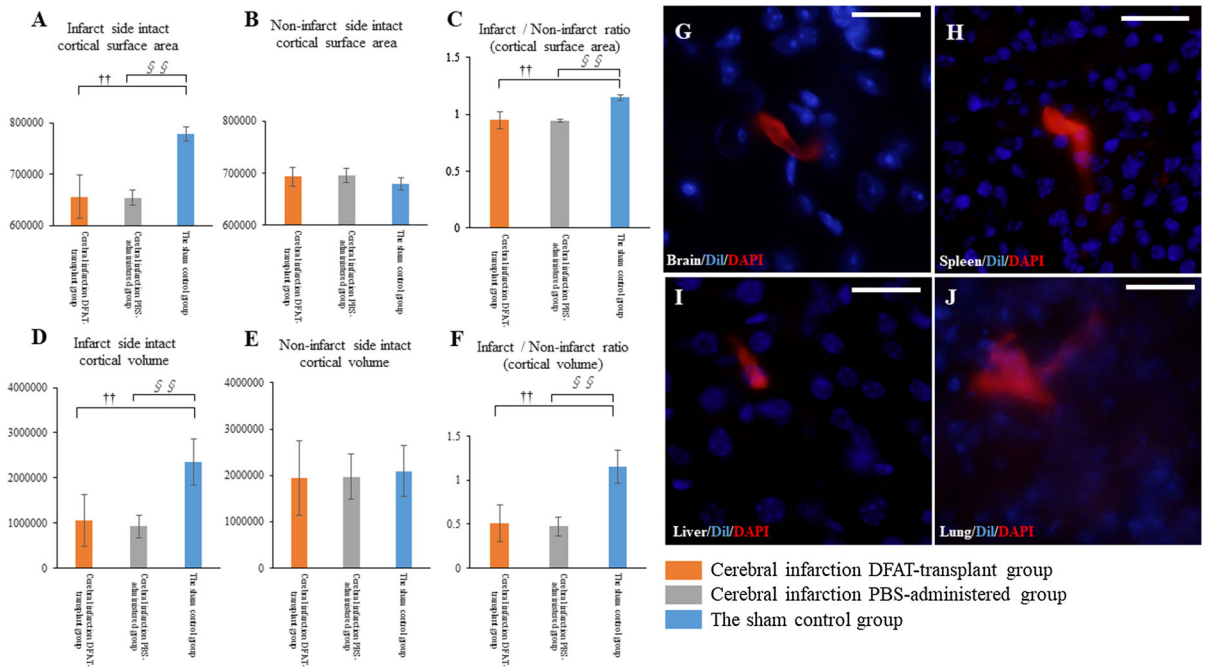

Fig. 5 Changes in cerebral capacity and transplant cell distribution due to DFAT transplantation. There was a significant difference between the cerebral infarction DFAT-transplant group and the sham control group, and between the cerebral infarction PBS-administered group and the sham control group in **a**, **c**, **d**, and **f**. No significant differences were observed among the three groups in the non-infarct side (contralateral cortex) (**b**, **e**). As a result of cell tracking with a Vybrant Multicolor Cell-Labeling Kit (DiI), labeled cells were observed in brain (**g**), spleen (**h**), liver (**i**) and lungs (**j**). *Scale bar* = 25 μm. ($^{\dagger\dagger}P < 0.01$ comparison between the cerebral infarction DFAT-transplant group and the sham control group. $^{\S\S}P < 0.01$ comparison between the cerebral infarction PBS-administered group and the sham control group)

angiogenesis, and oligodendrogenesis were promoted by MSC administration, and functional recovery after cerebral infarction was observed. Because DFAT have similar characteristic to MSC (Fig. 2), neurogenesis, synaptogenesis, angiogenesis, and oligodendrogenesis were promoted by DFAT transplantation, and functional recovery was observed in this study as well. However, we have not confirmed whether markers such as NF, Olig-2, SYP, and VEGF are expressed in the brain sections after DFAT transplantation. In order to elucidate by what mechanism DFAT promotes functional recovery, we would like to investigate these markers in a future study. Since mice in the sham group did not develop cerebral infarction, we speculate that there was no decrease in function, and the results of behavior tests were better compared with mice in the PBS-administered group or the DFAT-transplant group that developed cerebral infarction.

In the current study, we chose intravenous administration for cell transplantation, because this method is rather non-invasive for stroke-patient. However, after intravenous administration, labeled DFAT were distributed mainly in other organs, such as the spleen lungs and liver as well as the brain. Although a report has shown that intravenously administered human MSC reach their brains (Tfilin et al. 2010), others have noted that intravenously administered MSC are retained in the lungs, with a substantially lower percentage in the brain (Chen et al. 2001; Argibay et al. 2017). Conversely, a direct approach for transplantation to reach the affected area has been reported in studies with a depressed rat model and a murine spinal cord injury model (Gutiérrez-Fernández et al. 2013a; Chen et al. 2001). They showed that a sufficient number of cells reached the affected area, thereby confirming the effectiveness of the transplantation. In light of these reports, we should consider the method for transplantation other than intravenous administration, such as intra-arterial or intracerebral administration in the future.

In summary, we established DFAT from human buccal adipose pads and found that these cells had

characteristics similar to MSC in vitro. These cells are originally expressing neural stem cell markers before differentiation to mature cells and show neuronal differentiation with expressing some neural markers. Although the DFAT transplantation to the mice after cerebral infarction did not morphologically affect the post-infarct brain, it appears to improve some brain function estimated by behavioral tests. These findings suggest that the DFAT extracted from the patients' own tissue can be an option for the treatment of cerebral infarction.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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