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ORIGINAL ARTICLE

Neuronal differentiation potential of mouse mesenchymal stem cells derived from white versus brown adipose tissue

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Abstract: Mesenchymal stem cells (MSCs) from white adipose tissue (WAT) have a higher osteogenic differentiation potential than those from brown adipose tissue (BAT). The purpose of this study was to compare the neuronal differentiation potentials of these cells. In addition, to examine the applicability of these cells for autologous neuronal regeneration, the mouse hind limbs were assessed for neurological function after adipose tissue was harvested from the inguinal and interscapular regions. Adipose-MSCs were isolated from WAT of the inguinal subcutaneous regions and BAT of the interscapular regions in 6-week-old female C57BL/6J mice. The expression of cell-surface markers on the adipose-MSCs was examined by flowcytometry. The markers examined were CD90, Sca1, CD34, and CD45. Neuronal differentiation was assessed by immunocytochemistry using NeuN as a mature neuron marker. MSCs derived from WAT and BAT displayed similar immunophenotypes. Both cell populations differentiated into NeuN-positive cells (16.2% ± 1.0% of the WAT-MSCs and 14.7% ± 2.4% of the BAT-MSCs) in vitro. Harvesting the adipose tissue did not affect the neurological properties of the hind limbs. These findings indicate that both WAT-MSCs and BAT-MSCs may be attractive cells for autologous neuronal regeneration.

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Key words: Autologous; Brown adipose tissue; Mesenchymal stromal cells; Neuronal differentiation; White adipose tissue.

Introduction

Mesenchymal stem cells (MSCs) can be harvested from autologous donors and have the capacity to self-renew as well as the potential to differentiate into multiple lineages[1)]. These characteristics make MSCs very attractive for use in cell therapy and regenerative medicine[2)]. MSCs are found in various tissues, including bone marrow[3)], umbilical cord blood[4)], placenta[5)], and adipose tissue[6)]. Adipose tissue is an accessible, abundant, and reliable source for the isolation of adult stem cells suitable for tissue engineering and regenerative medicine applications[7)], and the treatment efficacy of adipose tissue-derived MSCs (AdMSCs) for neuronal regeneration in animal models has been reported[2, 8)].

There are two types of adipose tissue in mammals, white and brown. White adipose tissue (WAT) contains lipids that act as energetic fuel or as lipid reconstructive material, and brown adipose tissue (BAT) can use fatty acids for thermogenesis[9)]. WAT and BAT have different antigenic features and cell differentiation potentials, including osteoblasts, endothelial cells, adipocytes, hematopoietic cells, and cardiomyoblasts. In these differentiation potentials, BAT-derived cells display less plasticity[10)]. White adipose-derived stem cells can differentiate into neuronal and glial cells in vitro[11)]. Therefore, WAT has been thought to be more plastic than BAT and a more suitable source of stem cells[10)].

The subcutaneous inguinal WAT is located running ventrally across the femoral nerve[12-14)].

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So, harvesting inguinal WAT may affect the motor and sensory function. BAT may become a suitable cell source for autologous regeneration research instead of WAT. However, reports on the neuronal differentiation potential of MSCs derived from BAT are limited, and there are no studies that have compared the neuronal differentiation potential between WAT-MSCs and BAT-MSCs. It is also unclear whether harvesting inguinal subcutaneous adipose tissue and interscapular adipose tissue affects the motor and sensory function in mice.

The repair effects of MSC transplants depend on the degree of their survival[15]. Whether transplantations were autologous or allogeneic was important in transplantation experiments because cell survival rate was affected[16]. In allogeneic transplantation studies, MSCs could not survive even 1 month[15, 17]. However, MSCs could survive 3 months and could differentiate into neuronal and glial cells in an autologous transplantation study[18]. Autologous transplantation may promote the effect of MSCs thanks to long cell survival. Because adipose tissue is thought to be a suitable cell source for autologous transplantation as the clinical trial showed[2], the potential effects of its harvesting and the neuronal differentiation potential should be carefully assessed before pre-clinical studies are performed.

We hypothesized that BAT should be used as a cell source for autologous neuronal regeneration in pre-clinical studies. The goal of this study was to assess whether adipose tissues, WAT or BAT, are suitable for autologous neuronal regeneration. First, the neuronal differentiation potential of MSCs derived from WAT and BAT was compared. Second, whether the harvesting of inguinal and interscapular adipose tissue affected the neurological function of the hind limbs was investigated.

Material and Methods

Animals

Six-week-old female C57BL/6J mice were used as the source of adipose tissue (CLEA Japan, Inc., Shizuoka, Japan). All animal procedures were carried out in accordance with the guidelines of the institutional animal care and research advisory committee of Hirosaki University.

Isolation and culture of adipose tissue-derived MSCs (AdMSCs) from inguinal and interscapular adipose tissues

AdMSCs were isolated from adult mouse adipose tissues (n=60) as previously described with minor modifications[19]. WAT was dissected from the inguinal subcutaneous regions, while BAT was dissected from the interscapular region. The weight of the mice and resected adipose tissues was measured (n=10 each group). To confirm the identity of the WAT and BAT, each adipose tissue was assessed by HE staining. Adipose tissue samples were pooled in ice-cold DMEM, and then minced and digested with 10 ml of 0.2% collagenase type I at 37° C for 45 min. The resultant cell suspension was filtered through a 70-μm mesh to remove tissue debris, and then the collagenase was removed by centrifugation for 5 min. The cell pellet fraction was suspended in RBC lysis buffer (Becton Dickinson, Franklin Lakes, NJ, USA) to remove contaminating red blood cells. The cells were resuspended in DMEM supplemented with 10% fetal bovine serum (FBS), and then washed twice. The cells were then cultured with complete MesenCult™ (MesenCult MSC Basal Medium [Mouse] supplemented with serum-containing MesenCult MSC Stimulatory Supplements [Mouse], both from Stem Cell Technologies, Vancouver, Canada) supplemented with 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). After 24 h, the non-adherent cells were removed, and the

adherent cells were expanded by serial passage. When the cells reached 80~90% confluence, the adherent cells were detached with 0.125% trypsin and 0.01% EDTA and replated at a density of 5000 – 25,000 cells/cm^2. The live cell counts were performed with a TC20^{TM} Automated Cell Counter (Bio-Rad Laboratories, Hercules, CA, USA). The second passage (P2) adipose MSCs were used in the experiments (n=10 in WAT-MSCs, n=8 in BAT-MSCs).

Cell-surface markers of AdMSCs from WAT and BAT

Confluent mouse MSCs were analyzed using flowcytometry for the expression of cell-surface antigens CD90 (PE; Thermo Fisher Scientific), Sca1 (PE/Cy7; Becton Dickinson), CD34 (Alexa Fluor 647; Becton Dickinson), and CD45 (Alexa Fluor 700; Thermo Fisher Scientific). Two positive surface markers (CD90 and Sca1) and two negative surface markers (CD34 and CD45) were used to identify mouse adipose MSCs. Gating was set using rat monoclonal isotype antibodies which were used to detect any non-specific fluorescence, and in each analysis, at least 10000 events were collected. The cell-surface antigens of MSC markers were compared between WAT and BAT (n=3 in CD90, n=7 in Sca-1, CD34 and CD45).

Cell Separation of AdMSCs by magnetic-activated cell sorting (MACS)

To avoid stem-cell heterogeneity, MSCs were sorted by MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) before the assessment of their differentiation potential. AdMSCs were collected using Anti-Human/Mouse CD90-PE (1:100; Thermo Fisher Scientific). Anti-PE microbeads (1:2, Miltenyi Biotec) were used for the MACS separation of the MSCs. The target cell-labeled microbeads were trapped in a magnetic field and later collected as a positive fraction. MSCs isolated from WAT and BAT are hereafter referred to as WAT-MSCs and BAT-MSCs.

Neuronal differentiation potential of WAT- and BAT-MSCs

Neuronal differentiation was performed as previously described[20]. Experiments were repeated three times. Adipose-derived stem cells were harvested and transferred onto a sterile glass coverslip (1×10^4 cells/cm^2) for immunostaining. Cells were allowed to settle for at least 6 h and were then washed twice with serum-free DMEM. Neuronal differentiation was induced in Neurobasal medium (Thermo Fisher Scientific) containing 1% FCS, 1 × B27 supplement, 0.5 mM 1-methyl-3-isobutylxanthine, 1 μM dexamethasone, 50 μM 8CPT-cAMP, 10 mM valproic acid, and 10 μM forskolin. The neuronal differentiation medium was changed weekly. The cells were fixed with 4% paraformaldehyde in PBS for 30 min, and rinsed three times in PBS. The cells were blocked for 1 h with 10% (v/v) FBS, 3% (w/v) bovine serum albumin (BSA) in 0.1 M PBS (pH 7.4), 0.3 M sodium chloride, and 0.5% Triton X-100 (PBST), and incubated overnight at 4°C with primary antibodies in FBS+BSA+PBST. The cells were then washed three times with FBS+BSA+PBST, incubated for 1 h at room temperature with PBS/0.1% BSA containing secondary immunofluorescent antibodies, and washed three times with PBS. The cells were then incubated with murine-specific antibodies directed against NeuN (Thermo Fisher Scientific; Alexa-555), a marker of neuronal cells. The samples were inspected under a digital microscope system (BZ-X700, Keyence, Osaka, Japan). The total cells and NeuN-positive cells were counted under a microscope, and the ratio of NeuN-positive cells to total cells was determined.

Neurological assessment of hind limbs

A resection group, in which both WAT and

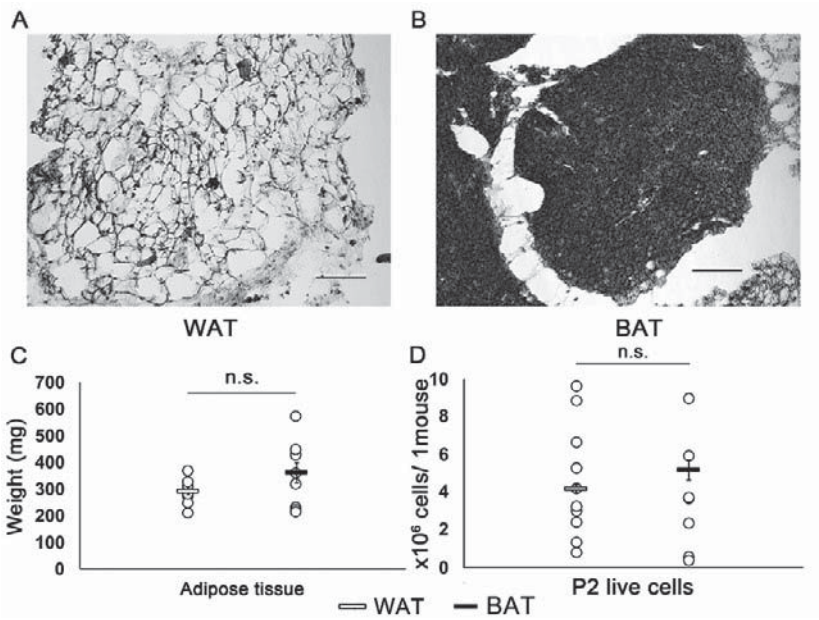

Fig. 1 Isolation and culture of AdMSCs from WAT and BAT

(A, B) HE staining of inguinal and interscapular adipose tissues. (A) Inguinal subcutaneous adipose tissue showed large fat droplets, which were defined as white adipose tissue (WAT). (B) Interscapular adipose tissue showed small fat droplets, which were defined as brown adipose tissue (BAT). Scale bars = 100 μm. (C) Comparison of the weight of WAT and BAT that were dissected from one mouse (n = 10/group). (D) Numbers of live cells in the passage 2 that were cultured per mouse (WAT n = 10, BAT n = 8).

BAT were resected, and a no resection (control) group were tested for hind-limb motor function and mechanosensitivity every week from six weeks of age (the time of surgery) until 12 weeks of age (n=8 at each time point). Two non-biased observers analyzed hind-limb performance using the Basso Mouse Scale (BMS) for locomotor scoring[21]. Motor coordination was also evaluated by a rotarod test at a speed of 10 rpm before resection and every 2 weeks after the adipose resection[22]. Hind-paw withdrawal thresholds (measured at 5 grams) in response to an innocuous mechanical stimulus were measured using a Dynamic Plantar Aesthesiometer set (Mechanical test)[23]. In this test, mice were placed in Plexiglas containers resting on an elevated glass surface. A mobile infrared emitter below the glass was placed under the center of the mouse's plantar hind paw, and activating the emitter started a timer[24]. The withdrawal latency was defined as the duration between the activation and termination of the infrared stimulus (measured in seconds); the final withdrawal latency was recorded as the mean of three measurements. A plantar test (heat test) was used to assess reactions to heat.

Statistical analysis

All data are presented as the mean value ± standard deviation (S.D.). Differences between two groups were evaluated for statistical significance using Mann-Whitney U test. Differences were considered significant at $p<0.05$.

Results

Isolation and culture of AdMSCs from inguinal and interscapular adipose tissues

Large fat droplets that were regarded as the WAT were seen more in inguinal subcutaneous regions than in interscapular regions (Fig. 1A). In interscapular regions, there were small fat droplets that were regarded as the BAT (Fig. 1B). The average body weight of the 6-week-old

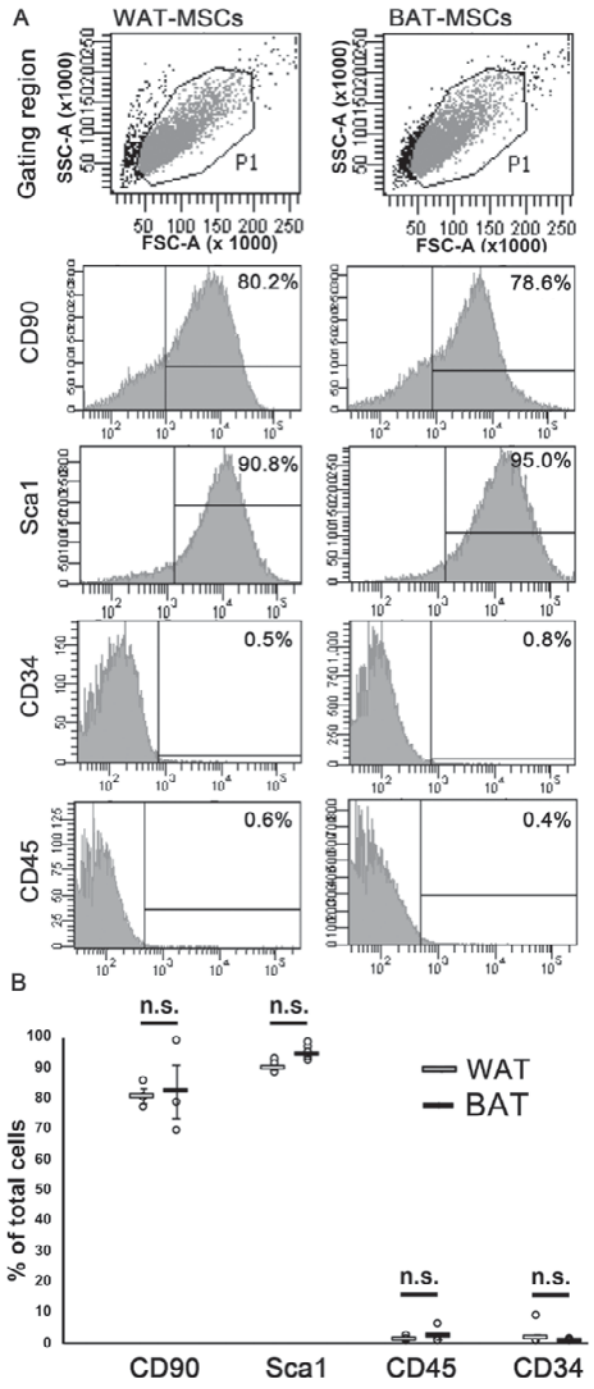

Fig. 2 Cell-surface markers of AdMSCs

(A) Typical flowcytometry profiles of white adipose tissue (WAT)-mesenchymal stem cells (MSCs) and brown adipose tissue (BAT)-MSCs. Rat isotype antibodies served as control and at least 10000 labeled cells were acquired. (B) Comparison of the cell-surface marker expression in MSCs derived from WAT and BAT (n=3 in CD90, n=7 in Sca-1, CD34 and CD45).

mice was 17.6 g ± 0.6 g. The average weight of the WAT was 296 mg ± 45 mg, and that of the BAT was 366 mg ± 118 mg. There was no significant difference in the weight of adipose tissue per mouse between WAT and BAT (p = 0.165; Fig. 1C). In P2, the number of adherent live cells was $4.5 \pm 3.0 \times 10^6$ cells/mouse from WAT, and $3.2 \pm 3.0 \times 10^6$ cells/mouse from BAT. There was no significant difference in the number of harvested cells per mouse between WAT and BAT (p = 0.32; Fig. 1D).

Cell-surface markers of AdMSCs from WAT and BAT

Flow cytometric analysis showed positive expression for CD90 and Sca1, and low expression of CD34 and CD45, indicating that cells derived from WAT and BAT had MSCs characteristics (Fig. 2A). Mean surface immunophenotyped expression values and standard deviations of WAT-MSCs and BAT-MSCs were as follows: CD90 (80.8 ± 2.6% and 82.3 ± 8.7%, respectively) and Sca1 (90.1 ± 1.8% and 94.6 ± 2.2%) and negative for CD34 (0.9 ± 0.8%, 2.0 ± 2.6%) and CD45 (1.4 ± 3.1%, 0.5 ± 0.3%) (Fig. 2B). The percentage of CD90-positive, Sca1-positive, CD34-negative, and CD45-negative cells showed no significant difference between WAT-MSCs and BAT-MSCs (p = 1.00, p = 0.089, p = 0.81, p = 0.32, respectively; Fig. 2B).

Neuronal differentiation potential of WAT- and BAT-MSCs

Both WAT-MSCs and BAT-MSCs differentiated into NeuN-positive cells (Fig. 3A). Cells positive for the mature neuron marker, NeuN, represented 16.2% ± 1.0% of the WAT-MSCs and 14.7% ± 2.4% of the BAT-MSCs (Fig. 3B). There was no significant difference in the percentage of NeuN-positive cells between WAT and BAT (p = 0.45; Fig. 3B).

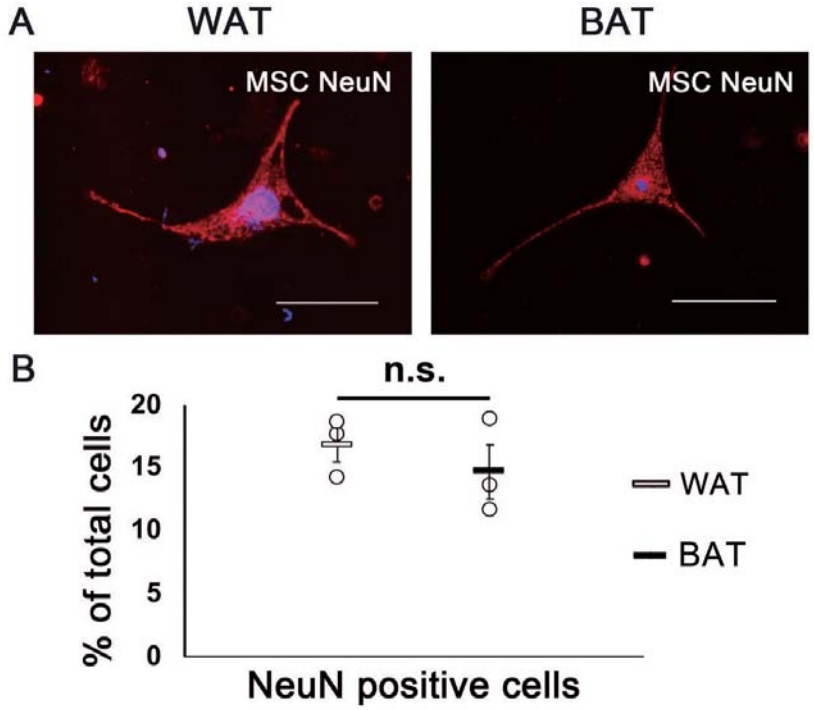

Fig. 3 Neuronal differentiation potential of WAT-MSCs and BAT-MSCs
(A) After neuronal induction, white adipose tissue (WAT)- and brown adipose tissue (BAT)-mesenchymal stem cells (MSCs) exhibited morphology similar to neuronal cells and were positive for NeuN. Scale bars = 100 μm. (B) Percentage of total cells that were NeuN-positive (n = 3/group).

Table 1. Neurological assessment of hind limbs

Week after resection			pre	2 weeks	4 weeks	6 weeks
BMS	(score)	resection	9 ± 0	9 ± 0	9 ± 0	9 ± 0
		control	9 ± 0	9 ± 0	9 ± 0	9 ± 0
Rotarod test	(sec)	resection	120 ± 0	119 ± 1.5	120 ± 0	119 ± 2.5
		control	117 ± 4.7	120 ± 0	117 ± 7.8	119 ± 2.0
		p-value	0.107	0.334	0.334	0.855
Dynamic plantar test	(g)	resection	5.7 ± 1.1	6.0 ± 0.8	5.3 ± 0.5	6.1 ± 0.9
		control	5.9 ± 0.9	5.4 ± 0.8	4.8 ± 0.8	5.9 ± 0.7
		p-value	0.625	0.154	0.135	0.591
Plantar test	(sec)	resection	6.6 ± 1.7	6.8 ± 1.2	6.4 ± 1.7	5.8 ± 1.9
		control	6.4 ± 1.7	7.6 ± 1.3	6.8 ± 1.5	6.8 ± 2.4
		p-value	0.776	0.213	0.553	0.365

The BMS (Basso Mouse Scale), Rotarod test, Dynamic plantar test, and Plantar test were analyzed 6 weeks after both white adipose tissue (WAT) and brown adipose tissue (BAT) resection. The averages were compared with the no resection (control) group. n=8 for each group.

Neurological assessment of hind limbs

The BMS score was 9 in both the resection and control groups (Table 1). The rotarod score was 119 ± 2.5–120 ± 0 seconds throughout the experimental period in the resection group, and 117 ± 4.7–120 ± 0 second in the control group (Table 1). In the mechanical test, the withdrawal thresholds throughout the experimental period were 5.3 ± 0.5–6.1 ± 0.9 g and 4.8 ± 0.8–5.9 ± 0.9 g in the resection group and control group, respectively (Table 1). In the heat test, the withdrawal latencies throughout the experimen-

tal period were 5.8 ± 1.9–6.8 ± 1.2 seconds in the resection group and 6.4 ± 1.7–7.6 ± 1.3 seconds in the control group (Table 1). Student's t-test showed no significant difference in any of the behavioral tests between the resection group and the control group. These findings indicated that the mice did not display any hind limb paralysis after undergoing the resection of both WAT and BAT.

Discussion

AdMSCs were isolated at around 4.0×10^6 cells/mouse from WAT in the inguinal subcutaneous regions, and 3.0×10^6 cells/mouse from BAT in the interscapular region in P2. The WAT- and BAT-MSCs differentiated similarly into mature neurons (16.2% in WAT-MSCs and 14.7% in BAT-MSCs). The adipose resection did not affect the neurological functions of the hind limbs.

In this study, WAT- and BAT-MSCs could be isolated from one mouse (Fig. 1D). Only a few studies have assessed the number of live cells isolated from one mouse, so it has been unclear whether enough MSCs could be isolated from one mouse for autologous transplantation. In previous studies on neuronal regeneration in mice, the number of transplanted no-induced-MSCs was 2.0×10^5 cells/mouse[25], and neuronal induced-MSCs was 2.5×10^5 cells/mouse[26]. Thus, our findings indicate that sufficient amount of AdMSCs is possible to be isolated for autologous neuronal regeneration in mice.

In a previous study, only a minority of the CD45-negative fraction was positive for CD90 and Sca1 in the BAT-stroma vascular fraction[10]. In the present study, MSCs derived from WAT and BAT displayed similar immunophenotypes: CD90(+), Sca1(+), CD34(−), CD45(−). Our method for preparing MSCs has several differences from those of the previous study, including the cell culture material, the number of passages, and the cell-surface markers used for flowcytometry. The Mesencult^{TM} medium, which was used in this study, is reported to be a good choice for AdMSCs, which exhibit a stable morphology and surface marker expression in this medium[27]. Using our methods, we were able to isolate and culture BAT-MSCs successfully.

This study demonstrated that there were no significant differences in the neuronal differentiation potential between WAT and BAT. A previous in vitro study showed that the osteogenic differentiation potential is lower in BAT than in inguinal WAT[10]. Adipocytes in the neck were generated from the neuroectoderm rather than mesoderm[28]. The difference between neuronal and osteogenic differentiation potentials may be caused by the origin (neuroectoderm or mesoderm) of the adipose tissue. WAT is also reported to be a more suitable source of stem cells than BAT[10]. Nevertheless, the present study revealed that BAT might be a useful source of MSCs that have a similar neuronal differentiation potential as those from WAT.

In many studies, inguinal adipose tissue, which is near the femoral nerve, is harvested after sacrifice of the mouse[13, 19, 26]. Because few studies have performed behavioral tests after adipose resection, it was unclear whether harvesting the inguinal adipose tissue affects hind limb function. Here we found that adipose resection did not affect the motor or sensory function in mice. Notably, motor and sensory behavioral tests are often performed to assess the effect of MSCs transplantation after spinal cord injury[25, 29]. Therefore, it was important to confirm that the resection of inguinal and interscapular adipose tissue itself does not affect the neurological assessment of the hind limbs of mice.

There were some limitations in this study. First, the MSC differentiation potentials were assessed only in vitro, and the findings still need to be confirmed by in vivo studies. Second, we

did not compare the glial differentiation potential between WAT-MSCs and BAT-MSCs. Finally, other behavioral and electrophysiological tests were not performed in this study. Because the WAT from inguinal adipose tissue is located near the femoral nerve, its dissection may affect some other tests of hind limbs.

In conclusion, we isolated AdMSCs from BAT, and found that BAT-MSCs and WAT-MSCs differentiated similarly into mature neurons in vitro. The harvesting of adipose tissue did not affect the neurological properties of the mouse hind limbs. These results suggest that both WAT and BAT are suitable as a cell source for autologous transplantation research.

Declaration of Conflicting Interests

The authors declare that there is no conflict of interest.

Acknowledgements

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