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Transplantation of mature adipocyte-derived dedifferentiated fat cells promotes locomotor functional recovery by remyelination and glial scar reduction after spinal cord injury in mice

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ABSTRACT

Mature adipocyte-derived dedifferentiated fat cells (DFAT) have a potential to be useful as new cell-source for cell-based therapy for spinal cord injury (SCI), but the mechanisms remain unclear. The objective of this study was to examine whether DFAT-induced functional recovery is achieved through remyelination and/or glial scar reduction in a mice model of SCI. To accomplish this we subjected adult female mice ($n = 22$) to SCI. On the 8th day post-injury locomotor tests were performed, and the mice were randomly divided into two groups (control and DFAT). The DFAT group received stereotaxic injection of DFAT, while the controls received DMEM medium. Functional tests were conducted at repeated intervals, until the 36th day, and immunohistochemistry or staining was performed on the spinal cord sections. DFAT transplantation significantly improved locomotor function of their hindlimbs, and promoted remyelination and glial scar reduction, when compared to the controls. There were significant and positive correlations between promotion of remyelination or/and reduction of glial scar, and recovery of locomotor function. Furthermore, transplanted DFAT expressed markers for neuron, astrocyte, and oligodendrocyte, along with neurotrophic factors, within the injured spinal cord. In conclusion, DFAT-induced functional recovery in mice after SCI is probably mediated by both cell-autonomous and cell-non-autonomous effects on remyelination of the injured spinal cord.

1. Introduction

Traumatic spinal cord injury (SCI) results in severe neurological deficits that can include paraplegia, but there is no effective clinical therapy to improve the locomotor function. Recently, the therapeutic efficacy from locomotor functional recovery following SCI have been reported in animal models by transplantation of several types of cells, such as embryonic stem cells, induced pluripotent stem cells, neural stem/progenitor cells (NPCs), and mesenchymal stem cells (MSCs) [1–6]. The mechanisms proposed for promoting locomotor function after SCI include: replacement of lost neurons to reconstruct local circuitry, remyelination of demyelinated axons by transplantation-derived oligodendrocytes, and the provision of trophic support that reduces the damage and glial scar to create a permissive substrate for axonal growth, etc. [1,2,5,6].

Mature adipocyte-derived dedifferentiated fat cells (DFAT) are one of the cell types studied for cell-based therapy for SCI. DFAT are fibroblast-like cells that sustain high proliferative activity and multilineage differentiation capacity similar to MSCs [7–9]. DFAT have several properties that are well suited to cell-based therapy as follows [9,10]: First, DFAT can be obtained from donors regardless of age. In our previous study using human DFAT from a total of 18 donors, ranging in age from 4 to 81 years old, we successfully prepared DFAT from every donor. Moreover, subcutaneous fat tissues can be easily collected from large numbers of healthy donors who undergo surgery or liposuction, because these tissues are typically discarded after surgery. From these perspectives, it is

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possible that DFAT provide a useful cell source for allogenic transplantation. Second, DFAT can be obtained from small amounts of tissue, because DFAT originate from a homogeneous fraction of mature adipocytes, the density of which is high in the tissue. Third, DFAT are highly homogenous. DFAT contain almost no other cell type even during first passage, because the cells are prepared from an isolated mature adipocyte fraction. This property of DFAT may not only lead to higher safety and efficacy for clinical cell therapies, but also be useful for mechanistic studies in cell-based therapy because of the small risk of contamination from other cell types.

Previously, we reported that DFAT transplantation had the efficacy to promote locomotor functional recovery in SCI rats [11]. However, it remains unclear whether the transplanted DFAT contributed to remyelination, glial scar reduction, and differentiated into a neural lineage after SCI. The aim of this study was to ascertain whether remyelination and reduction of glial scar by DFAT transplantation directly promotes functional recovery after SCI.

2. Materials and methods

2.1. Isolation and ceiling culture of DFAT

For analysis of DFAT survival after transplantation into the injured spinal cord, DFAT-Green Fluorescent Protein (DFAT-GFP) were prepared using a previously described method [7]. Briefly, mature adipocytes were obtained from the adipose tissue of GFP transgenic mice, from the Mitsubishi Kagaku Institute of Life Sciences. The tissues were minced and digested in collagenase solution (Collagenase Type II; Sigma) at 37 °C for 1 h under gentle agitation. After filtration and centrifugation at 135×*g* for 3 min, the floating top layer containing adipocytes was collected. After washing with phosphate-buffered saline, the cells were placed in culture flasks (BD Falcon 3107, Bedford) that were completely filled with Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical), supplemented with 10% fetal bovine serum (FBS; Moregate BioTech), and incubated in a humidified 5% CO_2 atmosphere. The cells floated and adhered to the top inner ceiling of the flask. Approximately 1 week later, the medium was removed and the flasks were turned upside-down so that the cells were at the bottom surface.

2.2. Spinal cord injury model and transplantation

All experiments were performed with the approval of the animal experiment committee of Nihon University (approval number: AP13B057). Spinal cord injury was induced at the Th10 level of adult female mice (n = 22, C57BL/6N, Charles River Laboratories Japan, 8 weeks old), using an Infinite Horizon Impactor (Precision Systems and Instrumentation; 60-kilodyne) under general anesthesia (isoflurane). The spinal cord was contused at a point on the midline, where the central canal was estimated to exist. The midline was confirmed by the anterior spinal vein, which was matched to a line connecting the spinous processes of vertebra at T9 and T11 levels. On the 8th day post-injury (dpi), SCI mice were randomly divided into DFAT and Control groups. In the DFAT group, 1×10^5 - DFAT-GFP in 2 μl were injected into the spinal cord at the Th10 level (lesion area) using a 26-gauge needle and a 10-μl Hamilton syringe (Sigma), which was vertically inserted using a stereotaxic injector (Muromachikikai) (0.5 μl/min). Controls received DMEM (2 μl) injections. Locomotor function score of hindlimbs, the Basso mouse scale (BMS) score [12], was assessed by two researchers at 1, 8, 9, 11 14, 17, 22, 29, and 36 dpi. When differences in BMS score between the right and left hind limbs were observed, an average of the two score was used. Any movements of mice during the sustained adduction of hind limbs were not scored.

In a preliminary study, to determine the timing of DFAT transplantation, we excised the contused spinal cord of mice, that were not used in this study, at 1, 3, 6, 9, and 14 dpi (Sup. 1A, n = 36), and performed histological analysis by hematoxylin and eosin (HE) staining (data not showed). In this preliminary study, the pathological condition until 6 dpi was mainly hemorrhage or cellular infiltration (may be inflammatory cells), and scar-like constitutions were observed after 9 dpi. Therefore, we decided to transplant DFAT on the 8th dpi, when the infiltration cells are low, and just before the appearance of a scar-like constitution.

2.3. Histological analysis

To reveal the potential mechanism of functional recovery by DFAT transplantation, we conducted histological analyses of the spinal cords at 36 dpi. Under deep isoflurane anesthesia, mice were intracardially perfused with 4% paraformaldehyde and the spinal cords harvested. Dissected spinal cords were post-fixed overnight in 4% paraformaldehyde, embedded in paraffin, and sectioned at 7 μm. After drying and deparaffinization processing, sections were subjected to Luxol fast blue (LFB) staining, HE staining, or immunohistochemistry (IHC). In IHC, the sections were incubated with the primary antibodies overnight at 4 °C, followed by antigen activation and blocking processes. The primary antibodies used were as follows: GFP (1:100, Santa Cruz), nestin (1:250, Santa Cruz), β III tubulin (1:250, Covance), neurofilament 200 kDa (NF-H, 1:5000, Aves Labs), glial fibrillary acidic protein (GFAP, 1:5000, Aves Labs),

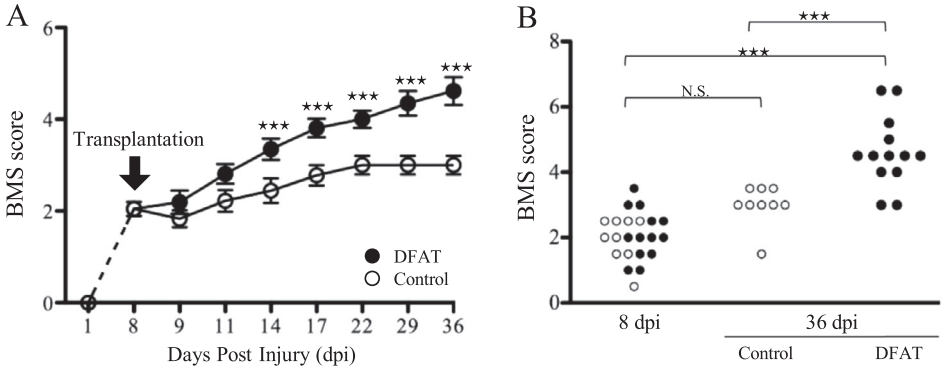

Fig. 1. Recovery of locomotor function by DFAT transplantation. (A) Comparison between pre-transplantation (8 dpi) and post-transplantation (9, 11, 14, 17, 22, 29, and 36 dpi). Mice in the DFAT group significantly recovered after 14 dpi compared with 8 dpi pre-transplantation. No significant difference in functional recovery was observed in the Control group. (B) Differences in the distribution of BMS score at 8 dpi and 36 dpi in the Control and DFAT groups. The locomotor function of mice in the DFAT group was significantly improved compared with the Control group. dpi, days post-injury. (DFAT group, n = 13; Control group, n = 9.) ***P < 0.001.

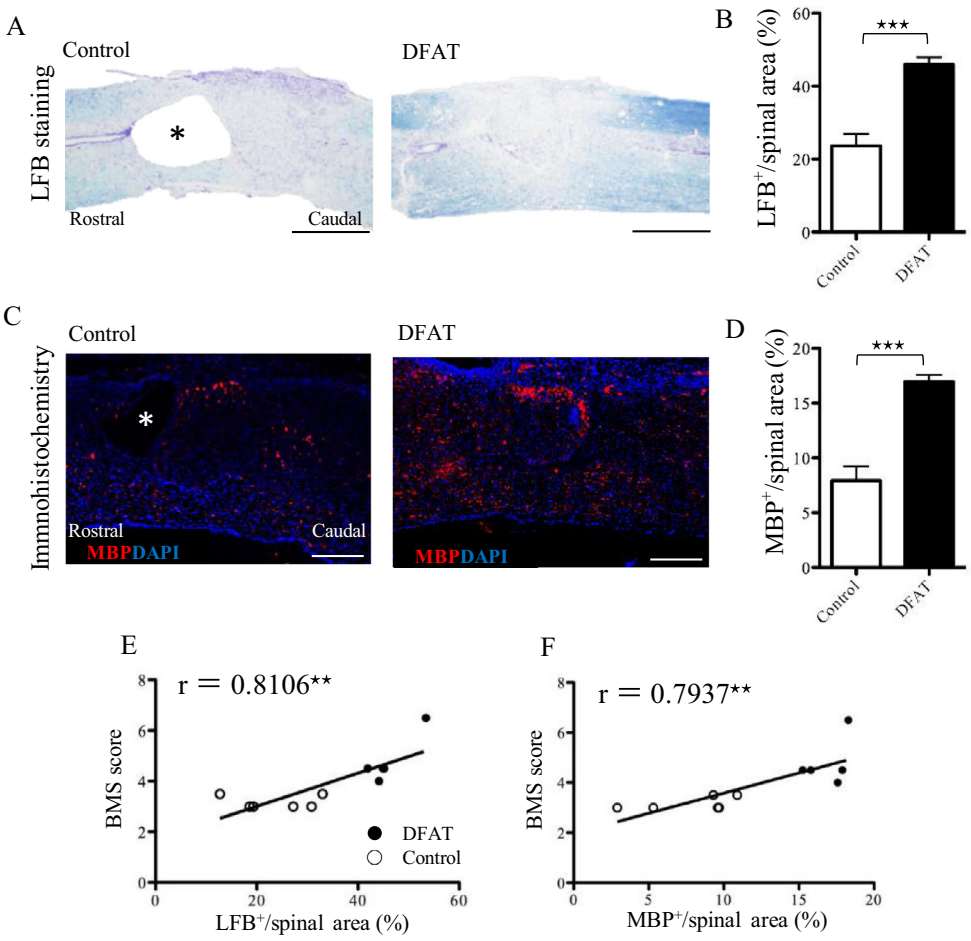

Fig. 2. Promotion of remyelination by DFAT transplantation in sagittal sections of the spinal cord at 36 days post-injury. (A) Images of LFB staining. The LFB-positive myelinated area (blue) in the DFAT group was larger when compared with the Control group. *Prominently extended central canal in the rostral part of the scar in the Control group. (Scale bar, 500 μm.) (B) The proportion of LFB-positive myelinated area to spinal area in the DFAT group was significantly higher compared with the Control group. (C) Expressions of MBP in immunohistochemistry. The MBP-positive myelinated area (red) in the DFAT group was larger compared with that in the Control group. (Scale bar, 250 μm.) (D) The proportion of MBP-positive area to spinal area was significantly higher in the DFAT group compared with that in the Control group. (E, F) Correlation analysis demonstrated a significant positive correlation with functional recovery in the LFB-positive and MBP-positive myelinated areas in injured spinal cords. LFB, Luxol fast blue; MBP, myelin basic protein. $^{**}P < 0.01$, $^{***}P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

adenomatous polyposis coli CC1 (APC, 1:250, Calbiochem), myelin basic protein (MBP, 1:2000, Aves Labs), brain-derived neurotrophic factor (BDNF, 1:200, Chemicon), glial-derived neurotrophic factor (GDNF, 1:500, Thermo Fisher Scientific Inc.), chondroitin sulfate proteoglycans (CSPG, 1:200, Sigma), CD11b (1:400, eBioscience). Sections were incubated in secondary antibodies (1:2000, Abcam/Molecular Probes) for 1 h at room temperature. In IHC, negative controls, without the primary antibodies, were used. Stained sections were photographed using a BZ-9000 microscope (Keyence). Spinal area, LFB-positive (LFB^+) area, MBP-positive (MBP^+) area and scar area, were quantified using Keyence analysis software (dynamic cell counter or measurement module; Keyence) ($n = 11$, each area). We used the midline sagittal images, where the rostral and caudal part of the central canals were observed, to quantify the parameters at the lesion epicenter. Regions used for quantification included the area encompassing 1.2 mm in the rostral and caudal direction from lesion epicenter (total 2.4 mm).

In the preliminary study described above, we also performed the assessments of locomotor function and histological analysis by LFB staining, with axial and sagittal sections of the spinal cord, to investigate the histological changes that accompanied with intrinsic recovery of locomotor function after SCI (Sup. 1, $n = 36$). In this preliminary study, the BMS score and the ratio of LFB^+ area to the spinal area (LFB^+/spinal area) showed high correlation coefficient with sagittal sections, but not with axial sections. Therefore, we used sagittal sections for all quantifications performed in this study.

2.4. Statistical analysis

Basso mouse scale score, spinal area, LFB^+ area, MBP^+ area, and scar area are presented as the mean ± SEM. All analyses were performed using GraphPad PRISM6 (GraphPad Software, Inc.). One-way analysis of variance (ANOVA) was used for comparisons of the BMS score between pre- and post-transplantation. An unpaired *t*-test was used for the comparisons between DFAT and Control groups (BMS score, LFB^+/spinal area, MBP^+/spinal area and scar/spinal area). The correlation between the parameters assessed (BMS score, LFB^+/spinal area, MBP^+/spinal area and scar/spinal area) was assessed with Pearson's analysis. A probability of less than 0.05 was considered significant.

3. Results

3.1. Transplantation of DFAT promoted locomotor functional recovery in SCI mice

We evaluated locomotor functional recovery in DFAT-transplanted mice using the BMS score. The SCI induced complete

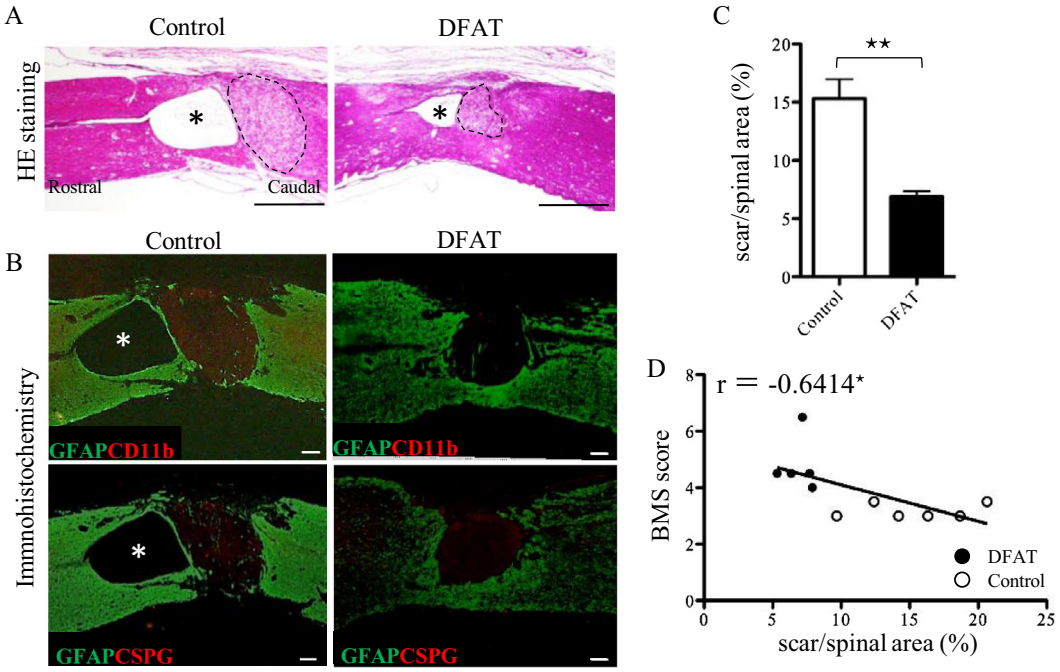

Fig. 3. Reduction of scar by DFAT transplantation in sagittal sections of the spinal cord at 36 days post-injury. (A) Images of scar stained with HE. Smaller scars were observed in the spinal cord of the DFAT group mice than in that of control mice. (Dotted line, scar; *extended central canal.) (Scale bar, 500 μm.) (B) Comparisons of CD11b and CSPG by immunohistochemistry. Positive areas of CD11b (red) and CSPG (red) in the DFAT group were smaller compared with those in the Control group in accordance with the reduction in scar size (GFAP-negative area). (Scale bar, 100 μm.) (C) The proportion of scar area to spinal area was significantly lower in the DFAT group compared with that in the Control group. (D) Correlation analysis demonstrated a significant negative correlation between BMS scores and the scar size. $^{\star}P < 0.05$, $^{\star\star}P < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

paralysis of hind limbs, followed by a gradual recovery. At 8 dpi, the mean BMS score was 2.0 ± 0.15 (Fig. 1A). At 14 dpi and beyond, significant functional recovery was observed in the DFAT group when compared with the BMS score at 8 dpi (Fig. 1A). No significant recovery in the BMS score was observed in the controls, between 8 and 36 dpi. Fig. 1B shows the distribution of the BMS score at 8 and 36 dpi. At 8 dpi, only 14% of SCI mice had a BMS score of 3. At 36 dpi, most of the DFAT mice had a BMS score of above 3, when compared to both the controls (36 dpi), and total mice at 8 dpi. Further, while functional recovery was limited to plantar placing using both their hind limbs (at 36 dpi) in the controls, no significance in BMS score was observed compared to 8 dpi. However, in DFAT group at 36 dpi, most of the mice managed to elevate their trunk with plantar stepping, using both their hind limbs (Video data). No significant correlation was found between the functional recovery (difference in BMS score between 8 and 36 dpi) and the severity of SCI (BMS score at 8 dpi), between the two groups (Sup. 2).

3.2. DFAT contributed to remyelination and reduction of glial scar after SCI

To reveal the potential mechanism of functional recovery by DFAT transplantation, we conducted histological analyses in sagittal sections of the spinal cords at 36 dpi. To evaluate the extent of remyelination after SCI, the proportions of LFB^+ and spinal areas were calculated. The LFB^+ proportion was higher in the DFAT group (Fig. 2A and B). To further confirm the increase in remyelinated area following DFAT transplantation, immunostaining was performed with antibody to MBP in the sections. The ratio of MBP^+ area/spinal area was higher in the DFAT group (Fig. 2C and D). This result was consistent with the positive correlation between the proportion of LFB^+ and MBP^+ (Sup. 3A). A significant positive correlation with functional recovery was observed in the LFB^+ ($r = 0.8106$) (Fig. 2E) and MBP^+ proportion ($r = 0.7937$) (Fig. 2F).

To examine another potential mechanism for functional recovery, the proportions of scar area/spinal area were calculated for evaluation of scar size after SCI. Upon HE staining, smaller scars were observed in the spinal cords of the DFAT group (Dotted line; scar) (Fig. 3A). Additionally, we performed immunohistochemistry with GFAP and CD11b (or CSPG) to further confirm the size of glial scar in the DFAT group (Fig. 3B). The boundary of the GFAP-positive area and GFAP-negative area was almost consistent with the outline of the scar observed in HE staining. In both groups, CSPG and CD11b mainly expressed in the GFAP-negative area, and around the scar, in both the groups. The ratio of scar/spinal area was significantly lower in the DFAT group (Fig. 3C) and the dilation of the central canal, which is associated with enhanced inflammation, astrogliosis, and apoptotic cell death [13], was not observed in the DFAT group compared with the Control group. Importantly, we observed a significant negative correlation between the scar proportion and BMS score ($r = -0.6414$) (Fig. 3D).

A significant negative correlation was found between the ratios of the scar, with either LFB^+, or with MBP^+ areas (Sup. 3B, C). When a comparison was made between the indices of functional recovery and the parameters of either remyelination or glial scar, functional recovery a relatively stronger correlation with the parameters of remyelination than with those of the glial scar reduction (LFB^+ proportion; $r = 0.8106$, MBP^+ proportion; $r = 0.7937$, scar proportion; $r = -0.6414$).

3.3. DFAT differentiated into neural cells and expressed neurotrophic factors in the injured spinal cord of mice

To investigate whether transplanted DFAT-GFP had differentiated into neural lineage and/or released neurotrophic factors, we performed immnohistochemistry on injured spinal cords at 36 dpi. Transplanted GFP-positive DFAT (DFAT-GFP) were detected mainly around the lesion epicenter in the DFAT groups (Fig. 4A and B). The DFAT-GFP derived-cells expressed APC (Fig. 4C) and MBP in

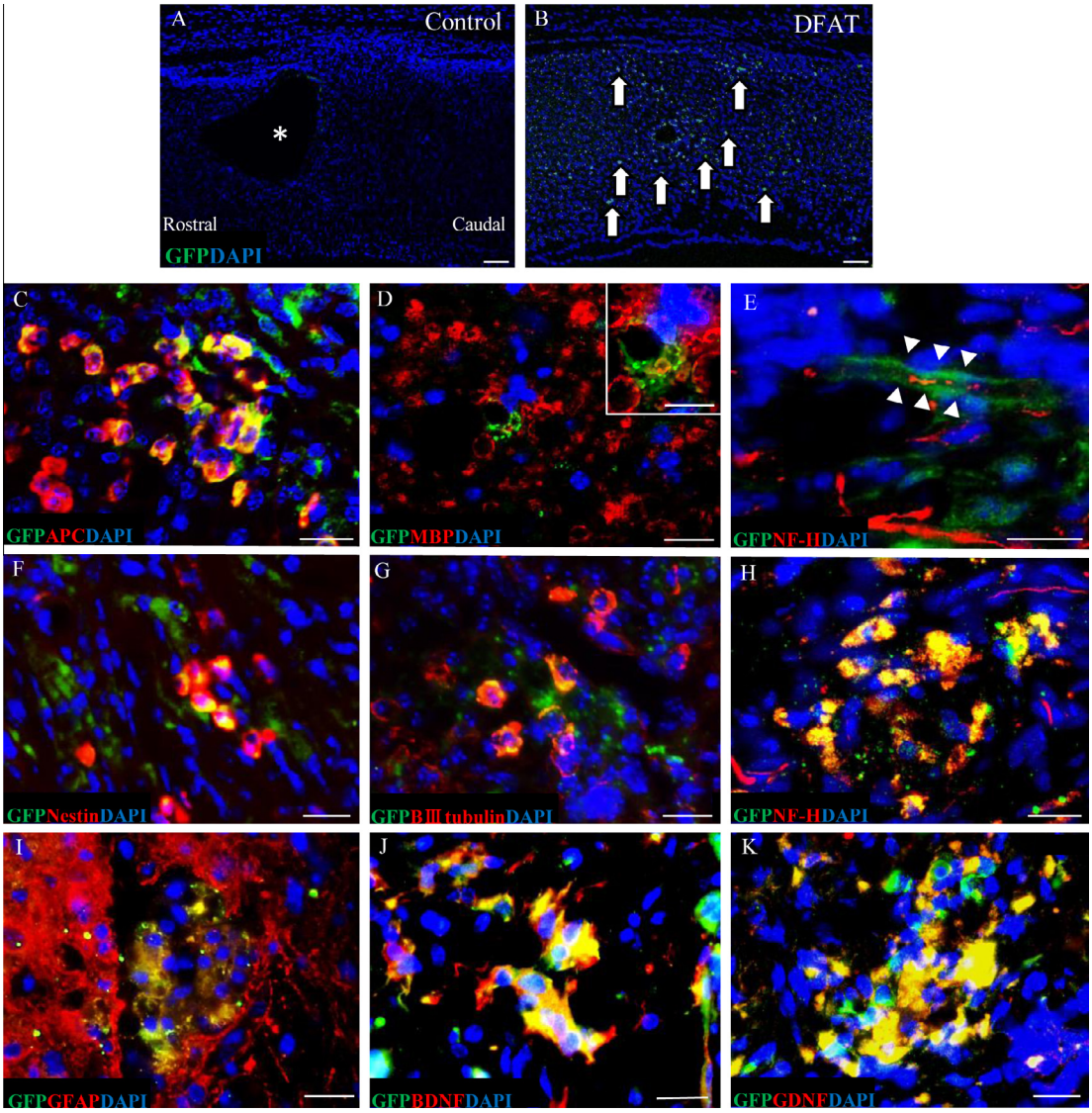

Fig. 4. Differentiation and release of neurotrophic factors by the transplanted DFAT-GFP in the injured spinal cord. (A, B) Intense DFAT-GFP (green) signal is seen in the DFAT group. (Arrows; detected DFAT-GFP. *extended central canal.) (C, D) The DFAT-GFP expressed markers of oligodendrocytes (APC, red) and myelin (MBP, red) in the DFAT group. (E) The DFAT-GFP showed the form like capsule, had a NF-H positive (red) neuron (arrowheads). (F–I) The DFAT-GFP expressed neural markers (red), nestin (F), β III tubulin (G), NF-H (H), and GFAP (I). (J, K) The DFAT-GFP expressed with markers of neurotrophic factors (red) in the DFAT group. (Scale bars: 100 μm in A and B, 20 μm in C–K, 10 μm in D inset.) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

a ring form (Fig. 4D), and the DFAT-GFP exhibited structures like myelin sheath that en-sheathed NF-H-positive neurons in the sagittal section (Fig. 4E). Additionally, DFAT-GFP derived-cells also expressed nestin, β III tubulin, NF-H, and GFAP (Fig. 4F–I), along with BDNF and GDNF (Fig. 4J and K).

4. Discussion

In the present study, we demonstrate that DFAT transplantation imparted functional recovery after SCI in mice, and this was accompanied by remyelination and glial scar reduction. Further, the transplanted cells integrated and differentiated into neural cells and oligodendrocytes, which myelinated axons. Additionally, the DFAT-derived cells expressed BDNF and GDNF, suggesting the possible release of neurotrophic factors in the injured spinal cords of mice.

For clinical application of DFAT transplantation in SCI patients, it would be necessary to elucidate the precise mechanism of functional recovery following transplantation, in an animal model. Theoretically, many possible mechanisms exist for the therapeutic effects achieved by the transplanted cells in SCI, including (a) a cell-autonomous effect; direct cell replacement for lost cells after SCI (e.g., forming new oligodendrocytes and/or neurons), and (b) a cell-non-autonomous effect; trophic support (indirect) to increase survival of host neural cells and host-mediated repair, regeneration, and/or plasticity [1,5,6,14].

In this study, the DFAT-induced remyelination contributed to promotion of functional recovery after SCI. Transplanted DFAT-GFP in the spinal cord expressed markers of mature oligodendrocytes and myelin, and exhibited structures like myelin sheath (Fig. 4C–E). These results strongly suggest that the remyelinating oligodendrocytes were indeed derived from the transplanted DFAT, and that it could directly contribute toward functional recovery by forming myelin sheath around demyelinated axons. The transplanted cells also expressed markers of NPCs, neurons, and astrocytes, suggesting sustained multi-potency, and differentiated into neural cells in the injured environment, in accordance with other stem cells [1,2,4]. All of this directly supports

the role of a cell-autonomous effect played by the transplanted DFAT.

Previous studies have demonstrated that remyelination after transplantation of NPCs contributes to functional recovery [6,15], as NPCs isolated from Shiverer mice – MBP null mice that do not form compact myelin – do not show the remyelination effect as NPCs from wild-type mice. In this study, promotion of remyelination contributed predominantly to functional recovery, more than glial scar reduction (Figs. 2E, F and 3D). This evidence suggests that remyelination, induced by transplanted DFAT, contributed significantly to recovery of locomotor function in SCI mice. However, in this study, there is a possibility that the DFAT stimulated endogenous remyelination.

Reduction of glial scar through cell therapy after SCI suggests that it creates a permissive environment for axonal growth and myelination, such as reduction of inhibitors of axonal extension, and contributes to functional recovery [16,17]. In studies of NPCs and/or MSCs, it was suggested that the transplanted cells released several factors, including neurotrophic (e.g., BDNF and GDNF), immunomodulatory (e.g., leukocyte inhibitory factor, LIF; transforming growth factor-β_1), or angiogenetic and anti-apoptotic (e.g., interleukin, IL-6; insulin-like growth factor I) factors, which were correlated with a reduction in lesion area [2,18–20]. In our previous and present studies we showed that DFAT released these factors including BDNF, GDNF, LIF, and IL-6 [10,11]. On the basis of this evidence, we believe that the reduction of glial scars in this study was achieved through the cell-non-autonomous effect of DFAT.

In summary, in this study we show that DFAT transplantation promotes remyelination, and inhibited glial scar formation in SCI mice, possibly through cell-autonomous and cell-non-autonomous effects. We propose that DFAT could be used as the donor cell source for effective therapy in SCI patients.

Conflicts of interest

We have no conflicts of interest to declare.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.082.

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