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.

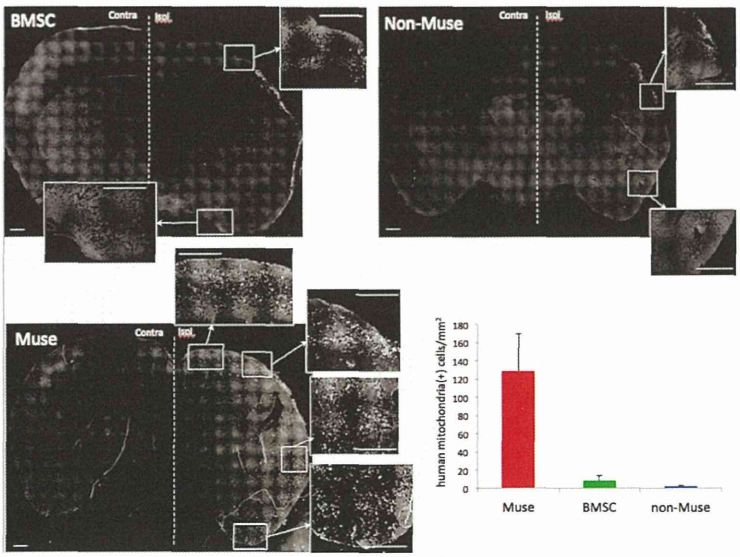


**Fig 4. Low-power photomicrographs of fluorescence immunohistochemistry using anti-human mitochondria antibody in BMSC-, non-Muse cell-, and Muse cell-treated mice at 42 days after transplantation.** A large number of human mitochondria-positive cells are engrafted in the peri-infarct area in Muse cell 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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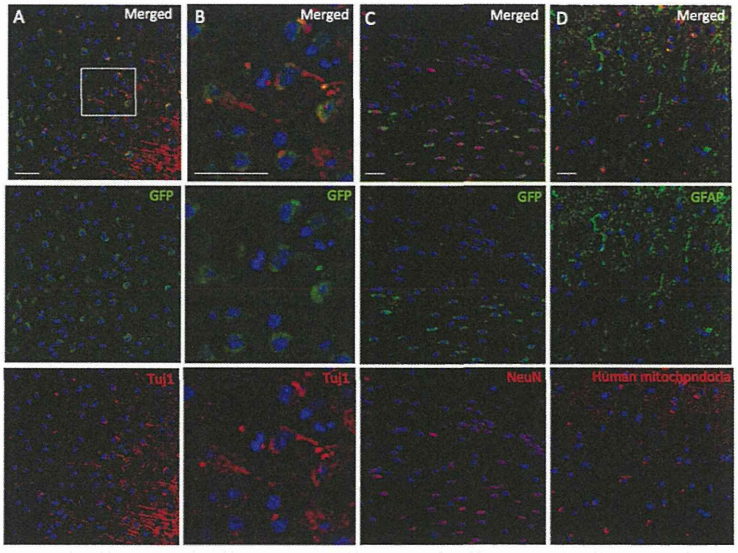


**Fig 5. Double immunohistochemistry of GFP-Tuj-1 (A and B), GFP-neuronal nuclear antigen (NeuN; C) and human mitochondria-GFAP (D) in ipsilateral cortex of Muse cell-group (42 days after transplantation).** The white square in panel A represents the location of Panel B. Scale bars = 50 μm.

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## Discussion

Although further analysis would be necessary, the present data provide important information on the mechanisms through which the engrafted BMSCs promote functional recovery after ischemic stroke. This study clearly demonstrates that at least two subpopulations in BMSCs,

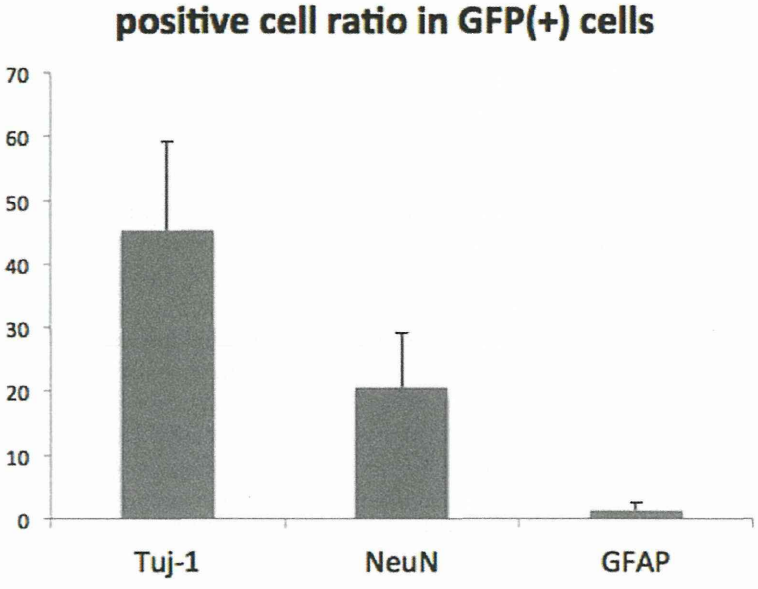


**Fig 6. Column graph shows the percentages of Tuj1-, NeuN- and GFAP-positive cells in GFP(+) cells in Muse group (42 days after transplantation).**

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Muse and non-Muse cells, may contribute differently to functional recovery when they are directly transplanted into the infarct brain. Non-Muse cells are considered to play a key role in early phase recovery of motor function after transplantation, because the non-Muse cell-transplanted animals started to recover motor function as early as 21 days after transplantation, while their therapeutic effects were limited and did not further promote functional recovery over 42 days. Histological analysis supports the findings. Immunostaining against human mitochondria detects negligible number of non-Muse cells, suggesting that they do not effectively remain or are not integrated into the host brain or even though they are once integrated, they might have disappeared by 42 days after transplantation.

On the other hand, Muse cell-treated animals did not show significant functional recovery during 28 days after transplantation, but started to recover motor function at 35 days post-transplantation, which was later than non-Muse cell-treated animals. On immunohistochemistry, however, a large number of human mitochondria- or GFP-positive cells were shown to engrafted and integrated into the peri-infarct area of ipsilateral hemisphere. There is possibility that some injected Muse cells were phagocytosed, while comparing remaining cell numbers in Muse, BMSC and non-Muse groups, Muse group showed substantial number of remaining cells with significant statistical difference to other two groups. Since cells were directly injected into the striatum by single shot, these integrated Muse cells might have migrated from the striatum into the peri-infarct area. Previous studies have shown that the transplanted cells aggressively migrate towards the lesion through the system of chemokines such as stromal cell-derived factor (SDF)-1 [24]. Majority of integrated Muse cells in the peri-infarct area expressed neuron-specific markers, Tuj-1 and NeuN at 42 days post-transplantation. Nearly 45% of GFP-labeled human Muse cells expressed Tuj-1, and about 20% of them were positive for NeuN, while the Muse cells doubly positive for GFAP was only ~1%, suggesting that Muse cells may preferentially differentiate into neuronal-lineage cells. We have previously shown that Muse cells express nestin, Musashi-1, NeuroD, and MAP-2 after neural induction, which correlates well with present results [14,29]. Longer evaluation of motor function is expected to find further improvement of motor function in Muse cell group.

Because of the sharp contrast between Muse cells and non-Muse cells in functional and histological analysis, it is most likely that these two BMSC subpopulations play biologically different roles in the infarct brain and contribute to post-stroke recovery of motor function and tissue repair by different ways and different timings. Since non-Muse cells did not remain in the host brain, early phase recovery observed in non-Muse cell group may result from the trophic effect exerted by transplanted non-Muse cells rather than cell replacement. In contrast, Muse cells are more responsible for replacement of the lost neuronal cells by integration into the host brain and spontaneous differentiation into neuronal lineage cells.

BMSCs that is consisted of several percentage of Muse cells plus vast majority of non-Muse cells significantly improved motor function by 21 days post-transplantation and yielded better therapeutic effects at 42 days than Muse cells and non-Muse cells. However, the number of integrated BMSCs was very small compared to Muse cells (Fig. 4). At least in short-term evaluation up to 49 days in functional recovery, the BMSCs look as the best to transplant because the mixture of biologically various subpopulations of cells may exert maximal therapeutic effects against ischemic stroke. Nevertheless, longer period observation would be necessary to evaluate therapeutic effects of Muse cells on motor function, because the number of BMSCs integrated into the brain was very small.

Very recent report has provided interesting information on the behaviors of BMSCs engrafted into the infarct brain. Fluorescence in situ hybridization (FISH) studies showed that about half of the engrafted BMSCs express mRNA for brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) at 14 days after transplantation into the infarct brain;

however, their percentages rapidly decreased thereafter. Instead, the percentage of microtubule-associated protein (MAP) 2–positive BMSCs gradually increased during 28 days after transplantation. These findings strongly support our results suggesting that the BMSCs may exhibit the trophic effect in the early (~2 weeks) stage of cell therapy and the phenotypic change toward neural cells thereafter, when transplanted into the infarct brain [23]. Previous reports have suggested that a certain subpopulation of transplanted BMSCs are integrated in the brain of rodent stroke model [2]. Since Muse cells have the ability to differentiate into neuronal cells both *in vitro* and *in vivo* while non-Muse cells do not show such neuronal differentiations[15], it is plausible small number of neuronal differentiation observed in BMSCs transplantation might be due to Muse cells.

Pathophysiological mechanism of post-stroke cognitive impairment is quite complicated [30]. However, recent studies have shown that the engrafted BMSCs may contribute to improve cognitive function in rodent models of chronic cerebral ischemia, although the underlying mechanisms are still unclear [10]. Indeed, both the BMSCs and non-Muse cells significantly increase the correct choice and decrease the error choice at 28 days after transplantation. Muse cells also decrease the error choice. Likewise the results in Rotarod test, longer evaluation of spatial memory may detect further improvement in the Muse cell-treated animals. It would be quite valuable to assess the mechanisms through which the donor cells recover cognitive function in animal models of CNS disorders. However, it should be reminded that the outcome measurements in animal experiments, including Rotarod and eight-arm radial maze test, may require further development. As recently pointed out by Balkaya et al. [31], the rotarod is a relatively simple and well-evaluated test for short-term (~4 weeks) evaluation of deficits after proximal MCA occlusion in a variety of mouse strains, but may depend on the other factors such as training protocol and motivation. Post-stroke cognitive function may also require several testing to determine the beneficial effects of cell therapy because of its complicated mechanisms.

As aforementioned, the Muse cells have high potential to become to neuronal cells because they differentiate into neuronal cells with very high ratio of ~90% under the presence of cytokine stimulation [14]. They have low telomerase activity and are non-tumorigenic. They comprise 0.03% of bone marrow mononucleated cells in bone marrow aspirate and several percentages of cultured BMSCs [32]. Wakao et al. (2014) have very recently suggested that Muse cells pay an exclusive role in trioblastic differentiation and tissue repair, while non-Muse cells do not directly participate in these events but rather have major roles in trophic and immunosuppressive effects [32]. These findings correlate well with the present histological data. However, the fact that the BMSCs include only several percentages of Muse cells may limit the beneficial effects of BMSC transplantation into the infarct brain. In other words, the therapeutic effects would be enhanced if the Muse cells are isolated or enriched and then transplanted into the infarct brain. Muse cells are quite attractive, because non-tumorigenic stem cells with the ability to generate the multiple cell type of the three germ layers can be obtained through easily accessible BMSCs without introducing exogenous genes [12,13,32]. Further studies are warranted to evaluate whether the therapeutic effect of BMSC transplantation can substantially be improved when Muse and non-Muse cells are combined at a certain best ratio.

## Conclusions

This study demonstrates that among BMSCs, Muse cells and cells other than Muse cells, namely non-Muse cells, may contribute differently to tissue regeneration and functional recovery when they are directly transplanted into the infarct brain. Substantial number of Muse cells remained in the host brain for up to 42 days and expressed neuronal markers Tuj-1 and NeuN,

suggesting they replaced the lost neurons, while only negligible number of BMSCs and non-Muse cells remained in the brain at the same time point. Muse and non-Muse groups did not show significant statistical difference in functional recovery, but Muse group was less potential than in BMSC group. Non-Muse cells, however, showed functional recovery at ~2 weeks, and thus they are speculated to exhibit trophic effects to improve the microenvironments of damaged brain at early stage. Since the proportion of Muse cells in BMSCs is only several percentage, tissue repair effect of BMSC transplantation would be enhanced when the ratio of Muse cells are increased, while trophic effect of non-Muse cells might also enhance therapeutic effect synergistically when combined with Muse cells with the best ratio.

## Author Contributions

Conceived and designed the experiments: MD SK. Performed the experiments: TY YK TM. Analyzed the data: TY YK TM SK. Contributed reagents/materials/analysis tools: TY YK TM HS KH. Wrote the paper: MD SK.

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