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# Paracrine Effect of Skeletal Muscle-Derived Stem Cell Transplantation: The Case of Peripheral Nerve Long-Gap Injury Therapy

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## ABSTRACT

The possibility and importance of paracrine effect of stem cell transplantation were discussed in this commentary. Although the primary purpose of stem cell transplantation therapy is absolutely the differentiation and incorporation of engrafted cells to facilitate the target tissue reconstitution. However, the paracrine effects surrounding recipient cells and/or donor cells each other is also a reasonable issue. We have experienced a typical case of paracrine effect in the experimental therapy of peripheral nerve injury using skeletal muscle-derived stem cells (Sk-SCs). The severe nerve injury with long-gap was made in the mouse and rat sciatic nerve, and bridged by a cellular conduit. The mouse Sk-CSs and bone marrow stromal cells (BMSCs) were obtained from GFP-Tg mice, and transplanted into the conduit. After 8-weeks, transplanted Sk-SCs differentiated into all the peripheral nerve support cells (such as Schwann, endoneurial/perineurial cells), and contributed to the recovery of the number of axon for almost 90% and close to 60% of myelin following significant functional recoveries. In contrast, BMSCs group showed the results similar to the non-cell transplanted control, and cells were eliminated during the first week. On the other hand, we also found that the human Sk-SCs, sorted as $CD34^{+}/45^{-}$ (Sk-34) cells, showed wholly comparable results of the mouse (such as cell engraftment, differentiation, and recoveries of axon/myelin, and functions)after 12-weeks of transplantation. However, the human $CD34^{-}/45^{-}/29^{+}$ (Sk-DN) cells, which was composed mostly skeletal-myogenic cells, showed no engraftment in the nerve tissue, but remained during 4 weeks, and showed significantly higher numerical and functional recoveries than the control, while these were clearly lower than Sk-34. This result suggested two important points; 1)the skeletal-myogenic cells are not able to grow and differentiate in the peripheral nerve specific niche, but 2) they accelerate nerve recovery, probably their paracrine effects. It is likely that during 4-weeks of cell survival with paracrine after transplantation is a sufficient condition, but one-week is insufficient.

**Keywords**: Sciatic nerve, Stem cell therapy, Schwann cell, Perineurial cells, Axon support cells.

## INTRODUCTION

The first and primary purpose of stem cell transplantation therapy is absolutely the differentiation and incorporation of engrafted cells and with a contribution to the target tissue reconstitution. However, the paracrine effects of transplanted cells are also important and should be considered to be a secondly purpose. Generally, analysis of paracrine/endocrine in vivo is difficult, because of its nature as the extracellular signaling form of cell-cell communication in which a cell produces a signal to induce changes in nearby cells. Signaling molecules, which are known as the cytokines (factors), are secreted by cells and diffuse over a relatively short distance. Secreted factors then travel in the interstitium, but the exact distance of it is not certain. Thus, their detection in the protein level is quite difficult. For this reason, the study focused upon the paracrine effects of stem cells in vivo is few.

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Recently, we experienced the certain case of the paracrine effects in the experimental therapies of the severely damaged peripheral nerve with a log-gap, using mouse and human skeletal muscle-derived stem cells (Sk-SCs). Because of this background, the paracrine effects of these cells before and after transplantation are discussed in this commentary, with the comparison with the case of bone-marrow-derived stromal cells (BMSCs).

Loss of vital functions in the somatic motor and sensory nervous systems can be induced by severe peripheral nerve transection with a long gap following trauma. In such cases, autologous nerve grafts have been used as the gold standard, with the expectation of activation and proliferation of graft-concomitant Schwann cells associated with their paracrine effects. However, there are a limited number of suitable sites available for harvesting of nerve autografts due to the unavoidable sacrifice of other healthy functions. To overcome this problem, we examined the potential of Sk-SCsas a novel alternative cell source for peripheral nervere generation therapy [1,2].The reason is that the Sk-SCs including $CD34^{+}/45^{-}$ (Sk-34) [3] and $CD34^{-}/45^{-}$ (Sk-DN) [4] cells, which showed differentiation potential into mesodermal cells (skeletal muscle cells, vascular smooth muscle cells, pericytes and endothelial cells) and ectodermal cells (Schwann cells and perineurial cells) in vivo, and typically exerted synchronized reconstitution of the muscular, vascular and peripheral nervous system in the severely damaged skeletal muscle[5-7]. The potential was not changed, when both Sk-34 and Sk-DN cells were used by the mixed cells, as the Sk-SCs [8-10]. Because of this background, we made sciatic nerve long-gap transection model of mice (7-mm) and rats (12-mm), and bridged using anacellular conduit made from separated esophageal submucous membrane from mice after 3-days of 70% ethanol treatment. Then, the cells were injected in the bridging-conduit [2]. As we expected in the mouse-to-mouse experiment, the transplanted Sk-SCs differentiated into all the peripheral nerve support cells(such as Schwann, endoneurial/perineurial cells), and contributed to the recovery of the number of axon for almost 90% and close to 60% of myelin (**Figure 1A and 1B**). Interestingly, myogenic cells were also observed in this nerve niche, but the skeletal myogenic capacity of expanded Sk-SCs had been diminished gradually, and completely disappeared at 4-weeks after the transplantation [2]. Similarly, BMSCs transplantation was also performed, however, the results showed almost no effects (wholly comparable to the level of medium control) for the recoveries of the number of axons and myelin (**Figure 1A and B**). In addition, transplanted BMSCs disappeared during first one week. Therefore, no cell engraftment with no effect is reasonable in this case.

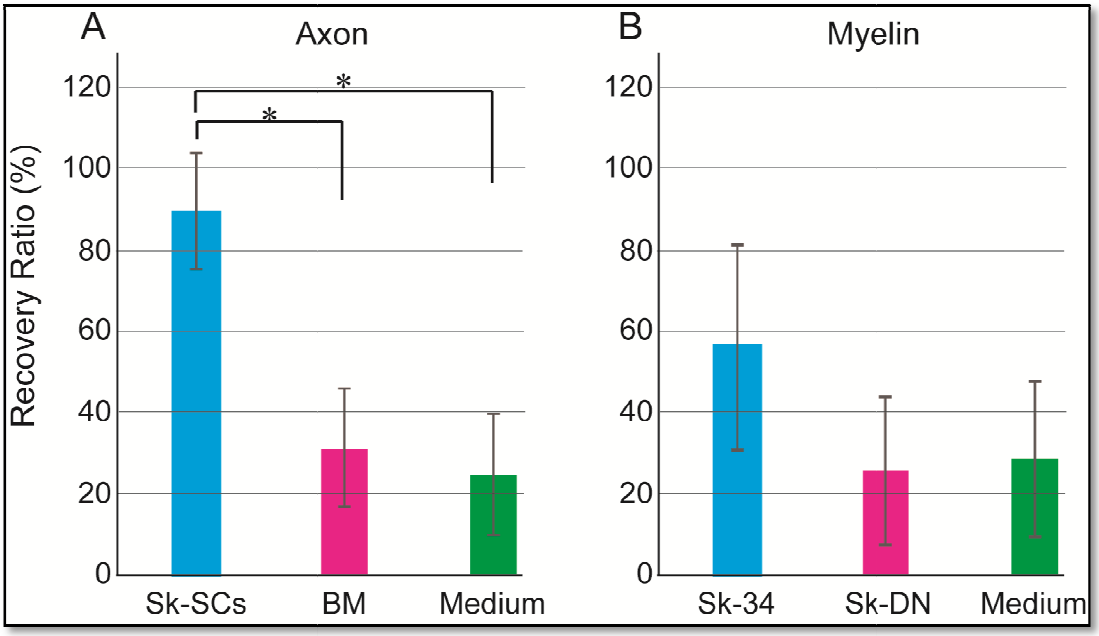



**Figure 1.** Recovery ratio of the number of axons (A) and myelin (B) in the bridging conduit at 8 weeks after mouse Sk-SCs and BM transplantation. The ratio was determined based on the normal control nerve and expressed percentages. Donor cells were obtained from Green fluorescent protein transgenic mice (GFP-Tg mice; C57BL/6 TgN[act EGFP]Osb Y01, provided by Dr. M. Okabe, Osaka University, Osaka, Japan) [12], and purified by previously reported method [5,6]. Detection of axon and myelin was performed using rabbit polyclonal anti-Neurofilament 200 (N-200, 1:1000, room temperature for 1 hour; Sigma, Saint Louis, MO), and by rabbit polyclonal anti-myelin basic protein (MBP; 1:200, room temperature for 2 hours; Millipore, Billerica, MA).Sk-SCs=skeletal muscle-derived stem cells, BM=bone marrow-derived stromal cells, Medium=medium transplanted control. *<0.05 by parametric Tukey-Kramer post-hoc test.

We also established the appropriate isolation and expansion culture method for the humanSk-34 and Sk-DN cells, and transplanted them into the sever muscle injury model of nude mouse and rat [11]. Then, we found that the combined results of both cell transplantation showed wholly comparable differentiation capacities of the mouse Sk-SCs [11]. However, very interestingly, the human Sk-DN fraction showed the limited inclusion of the skeletal-myogenic cells, whereas, the other multipotent stem cells were contained in the Sk-34 fraction [11]. Therefore, we applied human Sk-34 and Sk-DN cells to the nerve-gap model separately, because the elimination of skeletal-myogenic cells have been already confirmed in the previous mouse Sk-SCs experiment [2].As expected, the human Sk-34 cells showed cellular differentiations comparable to the mouse Sk-SCs (data not shown), with favorable recovery of the number of axons and myelin (**Figure 2A and 2B**), and the contractile functions of downstream muscles (**Figure 2C**) at 12-weeks after transplantation. More interestingly, human Sk-DN cells (skeletal myogenic cell dominant population) were also eliminated similar to the above case of BMSCs, but the term took 4-weeks after transplantation. A similar elimination of skeletal-myogenic cells in the nerve niche were also confirmed in the previous mouse Sk-SCs during 4-weeks after transplantation [2].Thus, it is certain that the skeletal-myogenic cell is not able to growth and differentiates in the peripheral nerve specific niche. In contrast, it is also clear that the Sk-DN cells remained during 4-weeks after transplantation, whereas the BMSCs was eliminated within one-week. In addition, the human Sk-DN group showed significantly higher regenerations than that of the medium control at 12-weeks after transplantation, while the levels are apparently lower than the Sk-34 group (**Figure 2A, 2B and 2C**).

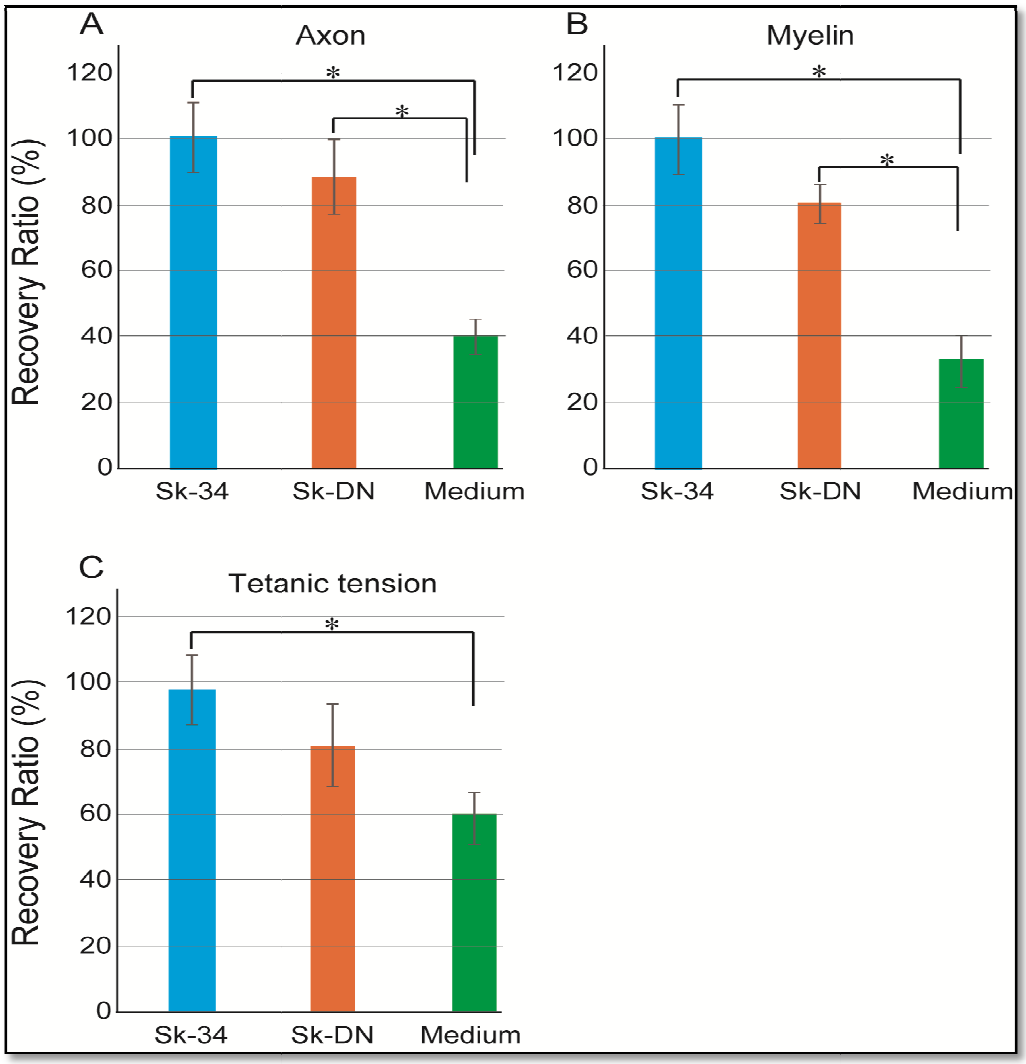
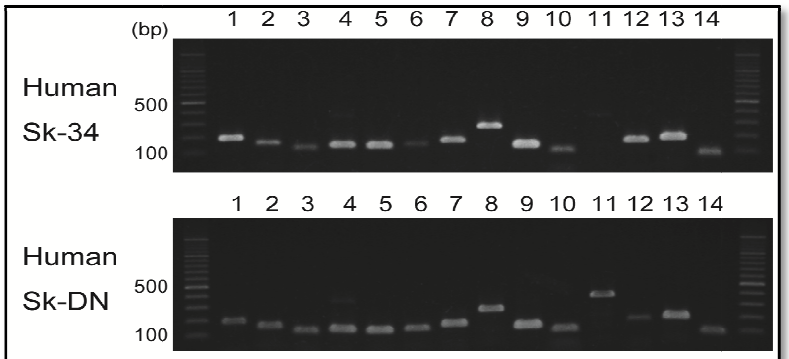



**Figure 2.** Recovery ratio of the number of axons (A) and myelin (B) in the bridging conduit, and tetanic tension output of the downstream muscles (C; total output of gastrocnemius, plantaris and soleus) at 12 weeks after human Sk-34 and Sk-DN cell

transplantation. The method of cell isolation, purification and expansion was reported previously [11], and the measurement of muscle tension output was also reported previously [5]. Detection of axon and myelin was performed as well as in Figure 1. *<0.05 by parametric Tukey-Kramer post-hoc test.

The observed differences in both groups are considered due to the prolonged survival/engraftment/paracrine of Sk-34 cells and their differentiation into all the nerve support cells. However, it is also certain that the complete elimination of the human Sk-DN cells occurred during 4-weeks after transplantation, but significantly higher morphological and functional recoveries than the control was achieved. Therefore, this is reasonable to consider that this result may be due to the paracrine effects of human Sk-DN cells during 4-weeks after transplantation. Putative paracrine capacities of human Sk-34 and Sk-DN cells, which was supposed by expressions of mRNAs were shown in **Figure 3**. Both cells consistently expressed 8 analyzed nerves and 5 analyzed vascular growth factors just before the transplantation, and a comparable paracrine effect can be expected. In fact, these expressions of mRNAs have wholly kept in the Sk-34 cells even at 4-weeks after transplantation; this was confirmed by the analysis for the engrafted Sk-34 cells, which was re-isolated enzymatically, and sorted using human-specific antibody (data not shown). In the comparison of the above mouse and human cell studies, both the mouse BMSCs and the human Sk-DN cells were eliminated in the peripheral nerve niche, but the former showed no effects for nerve regeneration, and the latter showed a significant contribution for the recovery. What makes a difference between them; that is the term of elimination, thus how long stay in the damaged tissue is considered to be the limiting factor of the paracrine effects following stem cell transplantation, because the expressions of nerve-vascular growth factor mRNAs were similar in both the mouse BMSCs [2] and present human Sk-DN cells (**Figure 3**) at just before the transplantation. In these view points, it is likely that one week of the cell retaining-period is insufficient, but 4-weeks retaining, with continuous expressions of growth factors after the onset of the damage, may have a beneficial effect on the nerve regeneration. However, we still not perform a more quantitative analysis (such as real-time RT-PCR) for the expressions of nerve-vascular growth factors in Sk-34 and Sk-DN cells. Therefore, further experiments, to clarify which factors may play a more prominent paracrine role during regeneration process, should be necessary.




| | | size (bp) | Fw | Rv |
|---|---|---|---|---|
| 1 | NGFb | 191 | TCCCTGCAGGACACACCATC | AAACAGCACACGGGGTGAAC |
| 2 | BDNF | 154 | GCAAACATCCGAGGACAAGGTG | GGTCCTCATCCAACAGCTCTTCT |
| 3 | GDNF | 119 | TTGCGATGCAGCTGAGACAAC | GGTCATCATCAAAGGCGATGG |
| 4 | Galectin1 | 141 | CCATCGTGTGCAACAGCAAG | ATCCATCTGGCAGCTTGACG |
| 5 | Ninjurin | 137 | CATCTTCATCACGGCCTTCG | CACCTCACAGGTATGGCGACTC |
| 6 | CNTF | 144 | CAGGTGCATTTTACCCCAACC | CATCCCATCAGCCTCATTGC |
| 7 | LIF | 169 | GCCTCCACAGCAAGCTCAAC | AGGAGTTGACAGCCCAGCTTC |
| 8 | FGFb | 269 | GAAGAGCGACCCTCACATCAAG | TCTGCCCAGGTCCTGTTTTG |
| 9 | VEGF | 140 | TCAGCGCAGCTACTGCCATC | ACTCCAGGCCCTCGTCATTG |
| 10 | PDGFb | 104 | GGGTTCCCTGACCATTGCTG | AGTTGGCGTTGGTGCGGTCT |
| 11 | EGF | 367 | ATGTCCCCTGTCCCACGATG | CCAAGGTTGAGGGCAAGAGG |
| 12 | HGF | 170 | TGCACGACAGTGTTTCCCTTC | CAGGCCTGGCAAGCTTCATTA |
| 13 | TGFb | 189 | CAATTCCTGGCGATACCTCAGC | AACCCGTTGATGTCCACTTGC |
| 14 | β-actin | 86 | GCCGAGGACTTTGATTGCAC | GGATGGCAAGGGACTTCCTG |

**Figure 3.** Expressions of nerve (1-8) and vascular (9-13) growth factor mRNAs in the human Sk-34 and Sk-DN cells just before transplantation. Lower stand shows analyzed factors with their primer sets and size. Detailed method was reported

previously [2]. β-actin was used for house-keeping control. Wholly the same expressions, except for EGF (No. 11), was observed in both cells, showing the putative paracrine capacities for the nerve and blood vessels. bp=base pair.

From the other point of view, it was also suggested that the paracrine substances of skeletal-myogenic cells exert facilitative effects for the peripheral nerve regeneration process. This concept further suggests that the skeletal muscle fiber and peripheral nerve regeneration process may share the large number of essential factors, and they co-working together. This notion also supports the previous results of Sk-SCs transplantation that asynchronized reconstitution of muscle-nerve-blood vessels were induced in the severe muscle injury with a sizable defect [5,6].

Finally, the cytokine supply associate with the stem cell transplantation was proposed in this commentary. However, administration therapy of recombinant cytokines, which will be selected appropriately in the near future, should be also considered. In particular, the nerve injury categorized in the Seddons's axonotmesis and/or the Sunderland's fourth degree, because the continuity of the epineurium (the most outer layer) is maintained, but involves loss of axons, endoneurial tubes, perineurial fasciculi and vascular networks, thus, associated with a bad prognosis. In addition, a development of the nerve bridging materials, which enable a sustained-release of cytokines, such as the biodegradable tubes, is also much-needed.

## CONCLUSION

Through these studies, it is supposed that the first 2-3 weeks from the onset of nerve damage may be a critical period to supply nerve-vascular growth factors to obtain the better nerve regeneration. This notion is found to be useful for future cytokine therapy for the severe peripheral nerve injury.

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