

SUCCESSFUL TRANSPLANTATION OF MOTONEURONS INTO THE PERIPHERAL NERVE DEPENDS ON THE NUMBER OF TRANSPLANTED CELLS

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ABSTRACT

Transplantation of motoneurons (MN) into the peripheral nerve to provide a source of neurons for muscle reinnervation, termed motoneuron integrated striated muscle (MISM), may provide the potential to restore functional muscle activity, when combined with computer-programmed functional electrical stimulation (FES). The number of MNs required to restore innervation to denervated muscles in adult Fischer 344 rats was investigated by comparing two groups, one transplanted with 2×10^5 cells (group A) and the other with 1×10^6 cells (group B). Twelve weeks after transplantation, electrophysiological analysis, muscle function analysis, and tissue analysis were performed. The mean motor nerve conduction velocity was faster (12.4 ± 1.0 m/s vs. 8.5 ± 0.7 m/s, $P = 0.011$) and the mean amplitude of compound muscle action potential was larger (1.6 ± 0.4 mV vs. 0.7 ± 0.2 mV, $P = 0.034$) in group B. The dorsiflexed ankle angle was larger in group B ($27 \pm 5^\circ$ vs. $75 \pm 8^\circ$, $P = 0.02$). The mean myelinated axon number in the peroneal nerve and the proportion of reinnervated motor end plates were also greater in group B (317 ± 33 vs. 104 ± 17 , $87.5 \pm 3.4\%$ vs. $40.6 \pm 7.7\%$; $P < 0.01$, respectively). When sufficient MNs are transplanted into the peripheral nerve, MISM forms functional motor units. MISM, in conjunction with FES, provides a new treatment strategy for paralyzed muscles.

Key Words: peripheral nerve, motoneuron, cell transplantation, muscle reinnervation, MISM

INTRODUCTION

Cell transplantation therapies have become a major focus of pre-clinical research as a promising strategy for the treatment of neurological disorders.¹⁻³⁾ However, the large number of cells required to reconstruct the complex structures of the central nervous system results in a high risk of tumorigenicity.⁴⁾ In addition, transplantation must be performed in the acute phase of an injury to avoid an enlarged cystic cavity at the injury site and glial scar formation.⁵⁾ As such, there are still several hurdles to overcome before the clinical application of neuronal replacement therapies is established for the central nervous system. One experimental approach to rescue denervated muscles from axonal disconnection or to lower motor neuron damage is the transplantation of

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motoneurons (MN) into the peripheral nerve to provide a source of neurons for muscle reinnervation. Since Erb *et al.* first reported the reinnervation of denervated muscle by embryonic MNs transplanted into the peripheral nerve,⁶⁾ several studies have investigated the factors that improve MN survival in peripheral nerves.⁷⁻⁹⁾ Considering the simplicity of the neural network and the wide window of opportunity for treatment,¹⁰⁾ the peripheral nerve system provides an ideal target for neuronal replacement therapy. This transplantation strategy, which we term 'motoneuron integrated striated muscle' (MISM), combined with computer-programmed functional electrical stimulation (FES), may provide the potential to restore functional muscle activity, even without any neural connection between the central nervous system and the muscle.¹¹⁾

Due to the high risk of tumorigenesis associated with transplanted cells, pluripotent cell-based regenerative medicine seems to be promising only in situations where transplantation of a relatively small number of cells can lead to sufficient functional restoration, such as in intraocular transplantation of stem cells for age-related macular degeneration.^{12,13)} In our previous study, only 1×10^6 embryonic cells were transplanted into the peripheral nerve after transection injury. We showed that approximately 1000 regenerated axons per nerve can generate manual muscle testing (MMT) grade 3 or higher muscle power using MISM technology.¹¹⁾ However, the number of MNs required to fully recover muscle function is still unknown. The purpose of this study is to determine the optimal number of MNs required for the successful restoration of denervated muscles by MISM technology.

EXPERIMENTAL PROCEDURES

Animal Model

All experimental protocols and animal maintenance procedures used in this study were approved by the Animal Ethics Research Committee at Nagoya University. Adult (8 week-old) Fischer 344 rats (Japan SLC, Inc., Shizuoka, Japan) were used as recipients and divided into two groups depending on the number of transplanted cells. Group A was transplanted with 2×10^5 cells and group B was transplanted with 1×10^6 cells ($n = 6$ for each group). One week after the sciatic nerve transection, E14 ventral spinal cord neurons were transplanted into the distal stump of the peroneal nerve. Twelve weeks after transplantation, electrophysiological analysis, muscle function analysis, and tissue analysis were performed.

Cell preparation

Ventral spinal cord cells were obtained from Fischer 344 rat embryos (Japan SLC, Inc., Shizuoka, Japan). Fischer rats on day 14 of pregnancy were anesthetized with isoflurane (2% delivered by a calibrated vaporizer through a facial mask) and their ventral spinal cords were resected using a surgical microscope and cut into small pieces in ice-cold Hanks' balanced salt solution. Ventral spinal neurons were dissociated using papain-containing dissociation solution (MB-X9901; Sumitomo Bakelite Co. Ltd., Tokyo, Japan) and were suspended in a Neurobasal medium containing B27 supplement, GlutaMAX, and N-2 supplement (all from Gibco Life Technologies, Tokyo, Japan).

Additional cell preparations were used to estimate the number of MNs present in dissociated ventral spinal cord cells.¹⁴⁾ Dissociated cells from ventral spinal cords were placed on Matrigel (356237 Matrigel Basement Membrane Matrix, Phenol Red-free; Corning Inc., NY, USA) thin-coated culture slides (354118 Falcon Culture Slides; Corning Inc., NY, USA), incubated at an initial density of 2×10^5 cells/cm², and were grown under standard conditions at 37 °C in 5% CO₂ for 16 hours. After incubation, the cells were fixed with warm 4% paraformaldehyde in

0.1 M phosphate buffer (pH 7.4). The presence of neurons and MNs was assessed using immunohistochemistry. Cells with a neuronal phenotype were detected with a mouse monoclonal anti- β -tubulin III antibody (T8660, 1:500; Sigma-Aldrich, MO, USA). MNs were detected with the MN-specific marker rabbit polyclonal anti-islet-1 antibody (AB4326, 1:300; Millipore, Billerica, MA). Fluorescently conjugated secondary antibodies were used to reveal the binding of primary antibodies (1:500; Invitrogen, Life Technologies, Tokyo, Japan). Hoechst 33342 (1:1000; Dojindo, Kumamoto, Japan) was used to selectively label the nuclei. For each preparation, the number of β -tubulin III positive and islet-1 positive cells was counted in 100 fields of cells from at least 10 different coverslips. MN ratio was calculated by dividing the number of cells stained with both β -tubulin III and islet-1 by the number of nuclei. In the dissociated ventral spinal cord cells, $82.9 \pm 2.1\%$ of the cells were positive for both β -tubulin III and islet-1. With the transplantation of 200,000 cells in group A and 1 million cells in group B, we estimate that an average of 165,826 and 829,132 MNs were transplanted in the respective preparations.

Surgical procedures and transplantation

All surgical procedures were performed under isoflurane anesthesia. The left sciatic nerve was completely transected at the mid-thigh. The nerve was ligated on both ends and the proximal nerve stump was sutured into the hip muscle to prevent reinnervation (Fig. 1A, B). One week after the sciatic nerve transection, E14 ventral spinal cord neurons were dissociated for transplantation. The cells were suspended in 10 μ l medium for transplantation. Using a Hamilton syringe with a 30G needle 2×10^5 cells in group A and 1×10^6 cells in group B were injected into the distal stumps of the peroneal nerves of rats (Fig. 1C). To confirm that the medium containing neurons was injected intraneurally, the nerve was examined under a dissecting microscope during injection to visually confirm swelling of the nerve. The injection site was 20 mm proximal to the entry into the tibialis anterior (TA) muscle. In a previous study, we demonstrated that neither neurons nor axonal growth was seen in rats that had undergone an identical surgery but had been injected with medium alone.¹¹⁾

Electrophysiological analysis

Twelve weeks after transplantation, electrophysiological analysis was performed. The compound muscle action potential (CMAP) of the TA muscle was measured at room temperature (24 °C) under isoflurane anesthesia, using a standard nerve-evoked potential recording system (Neuropack MEB-5504; Nihon Kohden, Tokyo, Japan). Two stainless steel monopolar recording electrodes (H537A; Nihon Kohden) were placed at the center of the belly of the TA muscle, one was placed at the insertion of the peroneal nerve, the other was placed 1 cm distal to the proximal one. The peroneal nerve was carefully exposed and a bipolar stimulating electrode (UM2-5050; Nihon Kohden) was placed around the nerve, 20 mm proximal to the entry into the TA muscle. Electrical pulses (supramaximal intensity; 100 ms duration; 1 Hz frequency; square wave) were applied with an isolator (SS-201J; Nihon Kohden) connected to an electronic stimulator.

Muscle function analysis

Muscle function analysis was performed following the electrophysiological analysis. Stainless steel wire electrodes (Bioflex wire AS633; Cooner wire, Chatsworth, CA, USA) were placed on the peroneal nerves and covered with a silicone tube and silicone gel for insulation and immobilization (Fig. 1D). The other ends of the wires were passed through the dorsal neck skin and connected to an electric generator (Neuropack MEB-5504; Nihon Kohden). After confirmation of ankle dorsiflexion by stimulating the peroneal nerves with trains of symmetrical biphasic square wave pulses (60 Hz), ankle angles formed by the lateral head of the femoral condyle, lateral

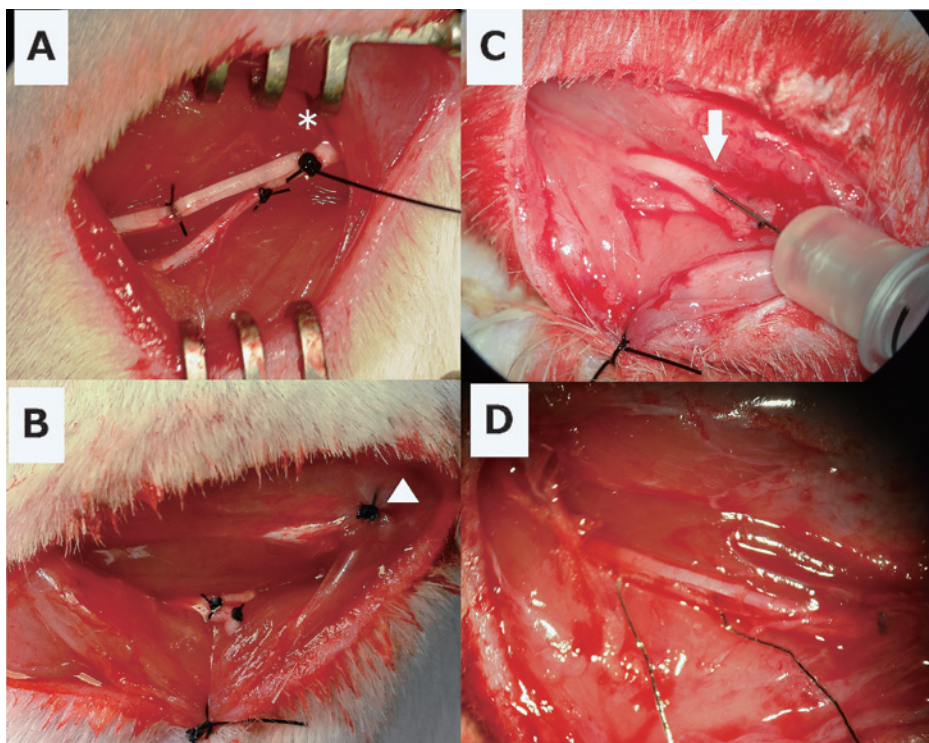


Fig. 1 Surgical procedure of nerve transection, cell transplantation, and installation of electrodes
 Photographs illustrating the surgical procedure of the sciatic nerve transection and cell transplantation into the peroneal nerve and installation of the electrodes.
 The nerve was ligated on both ends (A) and the proximal nerve stump was sutured into the hip muscle (B) to prevent reinnervation. One week after the sciatic nerve transection, E14 ventral spinal cord neurons were injected into the distal stumps of the peroneal nerves using a Hamilton syringe with a 30G needle (C). The injection site was 20 mm proximal to the entry into the tibialis anterior muscle. Twelve weeks after the transplantation, stainless steel wire electrodes were placed on the peroneal nerves and covered with a silicone tube and silicone gel for insulation and immobilization (D).
 Asterisks indicate proximal nerve stump. Arrowhead indicates the hip muscle with which the proximal nerve stump was sutured. Arrow indicates the injection site of the peroneal nerve stump.

malleolus, and the fifth metatarsal head were measured.¹⁵⁾ The stimulus intensity was increased from 0.0 mA in 0.2 mA increments to 3.0 mA, and the ankle angle at the different intensities was recorded.

Tissue analysis

The rats from both groups were perfused through the left ventricle with 50 ml 0.9% saline followed by 200 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The peroneal nerves and TA muscles were removed for immunohistochemical and histochemical analysis. The TA muscle was dissected free and immediately weighed, following which, the distal half of the TA muscles were embedded in paraffin, cut into 10- μ m thick cross sections, and stained with Alexa Fluor 488 conjugate wheat germ agglutinin (WGA, 1:1000; Molecular Probes, Eugene, OR, USA). The mean muscle fiber cross-sectional area at the middle of the muscle belly was then calculated. Five arbitrary points were automatically selected for calculation of the muscle

fiber cross-sectional area using an all-in-one type fluorescence microscope BZ-8000 (Keyence, Osaka, Japan). The proximal half of each TA muscle was analyzed for the formation of neuromuscular junctions (NMJ). The proximal muscles were cryoprotected in sucrose and frozen in dry-ice-cooled isopentane, after which 100 μm muscle sections were stained with a pan-neuronal marker (Milli-Mark FluoroPan Neuronal Marker, 1:500; Millipore) and Alexa Fluor 594 conjugate α -bungarotoxin (1:200, Molecular Probes, Eugene, OR, USA) in order to assess terminal muscle innervation. Z-serial images were collected by confocal microscopy (A1RSi, Nikon, Tokyo, Japan) and presented as single-plane projections. The percentage of reinnervated acetylcholine receptor (AChR) clusters, as determined by co-staining with the pan-neuronal marker, was determined using 5 microscopic fields per muscle.

The peroneal nerve distal to the transplant site was fixed in 4% glutaraldehyde in phosphate buffer. The extracted nerve was embedded in Epon; 1- μm thick sections were stained with toluidine blue (Sigma-Aldrich) for light microscopic examination, and the number and diameter of myelinated axons were measured using ImageJ (NIH, USA). Neuronal survival in the peripheral nerve was also examined. The transplant site of the peroneal nerve was cryoprotected in sucrose and then frozen in dry-ice-cooled isopentane. Peroneal nerve sections (10 μm thick) were stained with the antibodies against neurofilament H (1:500) and choline acetyltransferase (ChAT, 1:150; both from Millipore) in order to assess the survival of MNs. The sections were also stained with the antibody against glial fibrillary acidic protein (GFAP, 1:500; Millipore) in order to assess the survival of astrocytes.

Statistics

The Mann-Whitney U test, Student's t-test, and paired t-test were used where appropriate. All statistical analyses were conducted using the Statistical Package for Social Science version 19.0 software (SPSS Inc., Chicago, IL, USA). Values of $P < 0.05$ were considered statistically significant. All data are expressed as mean \pm standard error.

RESULTS

Electrophysiological analysis

Twelve weeks after transplantation, direct electrical stimulation of the left peroneal nerve evoked compound muscle action potential in the left TA and elicited ankle motion. CMAPs from the cell-transplanted side were evoked in all rats, even though no axonal connections to the central nervous system had been present for the preceding 12 weeks. The mean motor nerve conduction velocity (MCV) was faster (12.4 ± 1.0 m/s vs. 8.5 ± 0.7 m/s, $P = 0.011$) and the mean amplitude of CMAP was larger (1.6 ± 0.4 mV vs. 0.7 ± 0.2 mV, $P = 0.034$) in group B (Fig. 2A, B).

Muscle function analysis

The ankle dorsiflexion angle with electrical stimulation was 87 ± 8 degrees in group A and 46 ± 9 degrees in group B ($P = 0.005$), when a stimulus of 0.2 mA was applied (Fig. 2C). Moreover, as the stimulus current intensified, the ankle angle became increasingly dorsiflexed in group B, whereas it remained unchanged in group A. The ankle angles at maximum contraction were 75 ± 8 degrees in group A and 27 ± 5 degrees in group B ($P = 0.002$) (Fig. 2D). In our previous study, we showed a dorsiflexion angle in the naïve control group of 28 ± 3 degrees, similar to the angle in group B. These results demonstrate that transplanting approximately 83×10^4 MNs into the peroneal nerve (group B) generates MMT grade 3 or higher muscle power.

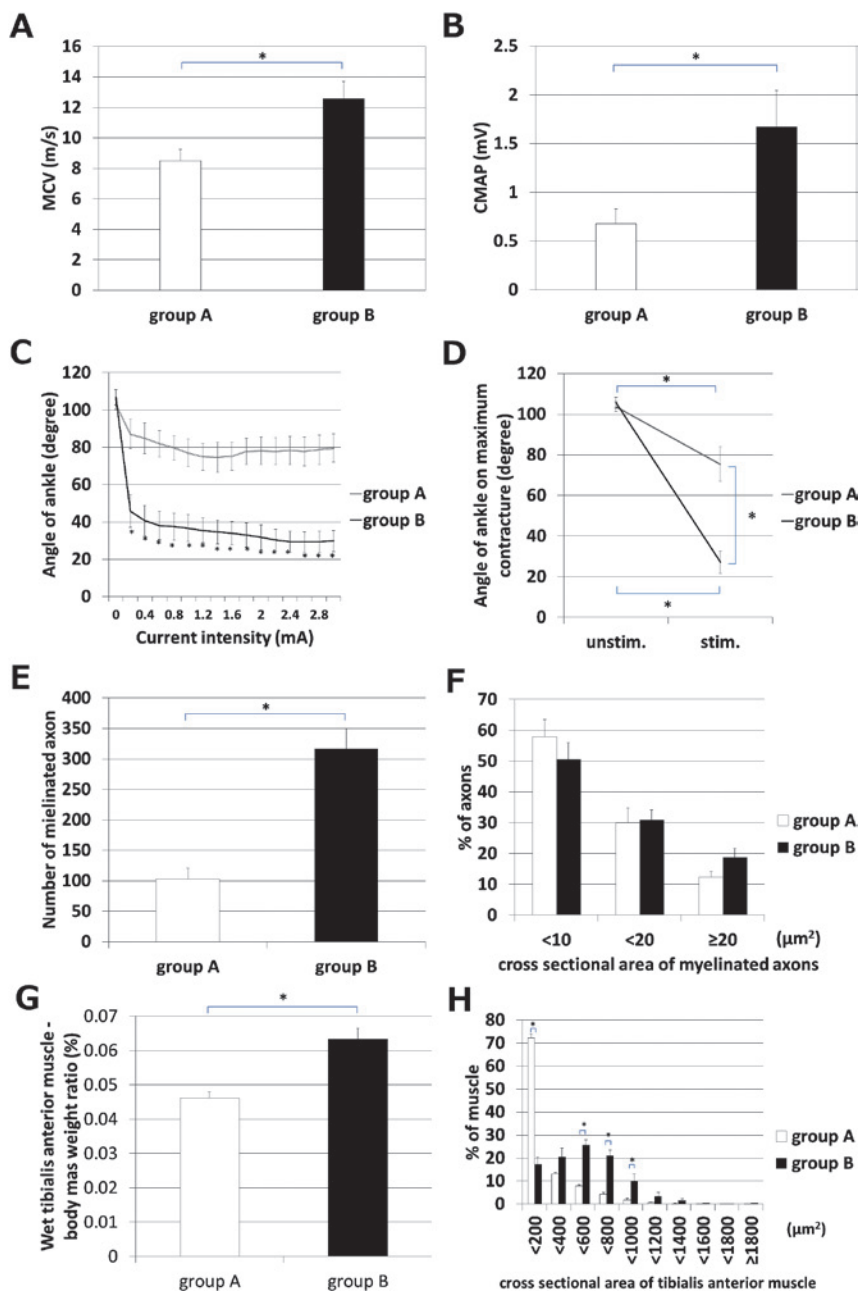


Fig. 2 Electrophysiological, muscle function, and tissue analyses

The mean motor nerve conduction velocity (MCV) (A) and amplitude of compound muscle action potentials (CMAP) (B) were recorded in the tibialis anterior muscle. The MCV was faster and mean amplitude of CMAP was larger in group B. Ankle dorsiflexion angle caused by electrical stimulation with varying current intensity (C) and ankle angle with and without electrical stimulation (D) were measured. The number of myelinated axons in the peroneal nerve (E) was greater in group B, while there was no difference in the cross-sectional area of myelinated axons (F). The weight of tibialis anterior muscle (G) and muscle fiber cross-sectional areas of the tibialis anterior muscle (H) were also greater in group B. Bar and line graphs represent mean \pm standard error. (n = 6 per group). *p < 0.05.

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Tissue analysis

Staining with toluidine blue confirmed the presence of myelinated axons in the left peroneal nerves of both groups (Fig. 3A, B). The mean number of myelinated axons was 104 ± 17 in group A and 317 ± 33 in group B ($P < 0.001$) (Fig. 2E). The mean cross-sectional area of the myelinated axons in the peroneal nerve was $11 \pm 0.8 \mu\text{m}^2$ in group A and $13 \pm 1.2 \mu\text{m}^2$ in group B ($P = 0.145$). A histogram according to area division showed a similar distribution between the two groups (Fig. 2F). Transplanted MNs and their axons stained with the antibodies against neurofilament-H and ChAT were observed in the transplant sites of the peroneal nerve of all rats in both groups (Fig. 3C). In addition, immunofluorescent staining with anti-GFAP antibody detected transplanted astrocytes at the transplant site in the left peroneal nerves (Fig. 3D). Both

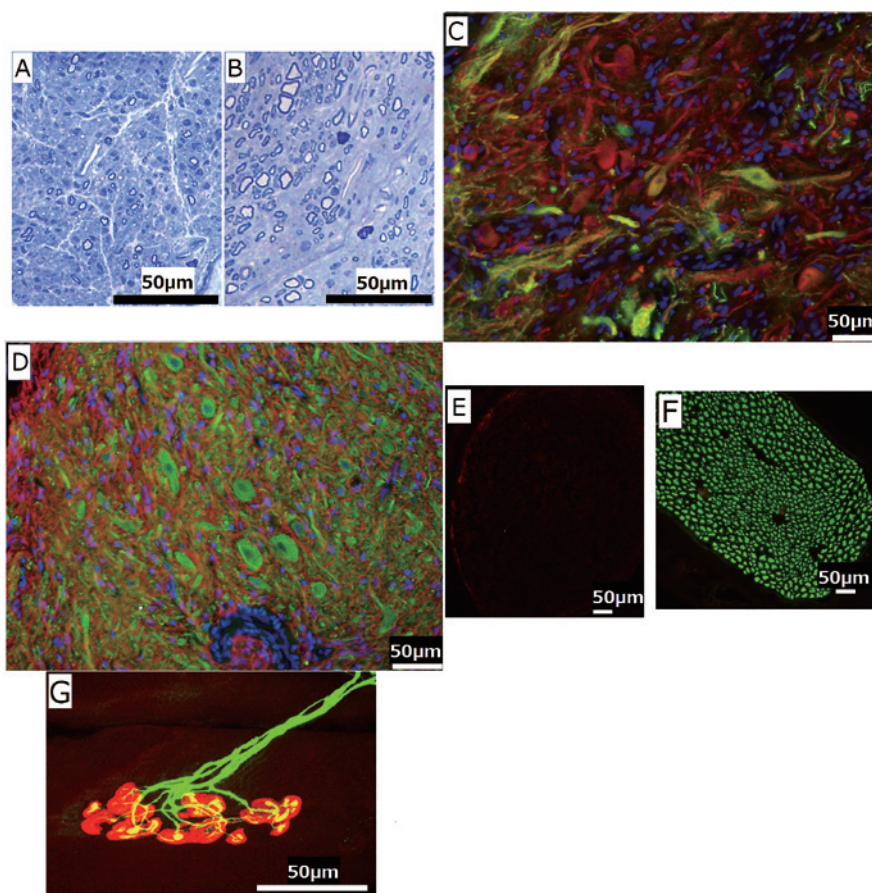


Fig. 3 Myelinated axon, motoneuron, and astrocyte survival in the peroneal nerve and the formation of neuromuscular junctions in the tibialis anterior muscle

Peroneal nerve sections stained with toluidine blue show lesser number of myelinated axons in group A (A) than in group B (B). Motoneurons (C) and astrocytes (D) survived in the peroneal nerve for 12 weeks. Green = choline acetyltransferase; red = neurofilament H (C). Green = neurofilament H; red = glial fibrillary acidic protein (D, E, F). Neurons and astrocytes were not present in surgical control (E) and naïve (F) groups; only axons were stained in the naïve (F) group. Formation of neuromuscular junctions was confirmed in the tibialis anterior muscle. Green = pan-neuronal marker; red = α -bungarotoxin (G). Scale bars = 50 μm .

MNs and astrocytes survived in the peripheral nerve for 12 weeks.

The mean wet muscle weight of the TA was greater in group B than in group A ($0.063 \pm 0.003\%$ BW vs. $0.046 \pm 0.002\%$ BW, $P = 0.001$, Fig. 2G). Muscle fiber cross-sectional area also was larger in group B ($187.0 \pm 7.3 \mu\text{m}^2$ vs. $512.3 \pm 45.8 \mu\text{m}^2$, Fig. 2H). NMJ formations were confirmed in the TA muscle of all rats that received cell transplants. Figure 3G shows motor endplates in a reinnervated TA muscle at high magnification. Regenerating axons stained with antibodies against neurofilament proteins reached the fields of motor endplates, as indicated by α -bungarotoxin labeling of acetylcholine receptor clusters. The reinnervation was assessed by the percentage of AChR clusters that an axon reached. Compared to group A, group B had a greater proportion of reinnervated motor endplates ($40.6 \pm 7.7\%$ vs. $82.1 \pm 5.9\%$, $P = 0.005$, Fig. 4).

DISCUSSION

This study demonstrates that transplanting approximately 83×10^4 MNs into the peroneal nerve can generate MMT grade 3 or higher muscle power with electrical stimulation and that transplanting a smaller number of MNs results in insufficient recovery in the denervated muscle. Although the number of regenerated myelinated axons was much smaller than that of transplanted MNs, only 317 myelinated axons in the peroneal nerve were required to restore functional muscle activity using MISM and FES. We also show that not only transplanted MNs, but also transplanted astrocytes survived in the peripheral nerve for 12 weeks.

A considerable number of MNs transplanted into the peripheral nerve was necessary for functional recovery of the denervated muscle. Our results are not in line with those reported by Grumbles *et al.*, which showed that muscle reinnervation and function were not limited by the number of MNs that were transplanted into the peripheral nerve.¹⁴⁾ They used approximately 70 thousand MNs from 1 million ventral cord cells and 140 thousand MNs of 200 thousand purified ventral cord cells. They concluded that transplantation of more MNs did not improve neuronal survival and functional recovery. This discrepancy may be partly explained by the fact that, in their study, the proportion of MNs or other ventral cord cells, such as glial cells, varied across the two groups. Mean MN survival was significantly higher for ventral preparations (3.4%) versus purified preparations (1.5%). This may indicate that supportive cells are needed for MNs to survive in the peripheral nerve since neurons do not naturally exist there. Our observation that transplanted astrocytes survived in the peripheral nerve may support the view that transplanted glial cells are important to optimize the intraneural environment for MNs.

There have been many difficulties in central nervous system reconstruction due to its complex neural network, requiring a considerable number of cells and a narrow time window for successful cell transplantation, even as many tissue engineering studies are conducted on the brain or spinal cord.^{10, 16)} The larger amount of cells required for transplantation may increase the risk of tumorigenicity. The total number of neocortical neurons in the normal human brain is estimated to be 19 billion in females and 23 billion in males,¹⁷⁾ whereas the number of motor units in a peripheral nerve is usually several hundred to several thousand.¹⁸⁾ Theoretically, a much smaller number of MNs is required for peripheral nervous system reconstruction. By improving neurogenic niches, we think that the optimal amount of transplant cells needed to obtain functional recovery with MISM can be further reduced.

The therapeutic time window is considered narrow in central nervous system reconstruction. Because the immediately post-traumatic microenvironment of the spinal cord is in an acute inflammatory stage, it is not favorable for the survival and differentiation of neural stem cell transplants. In the chronic stage after injury, glial scars form in the injured sites, which inhibit

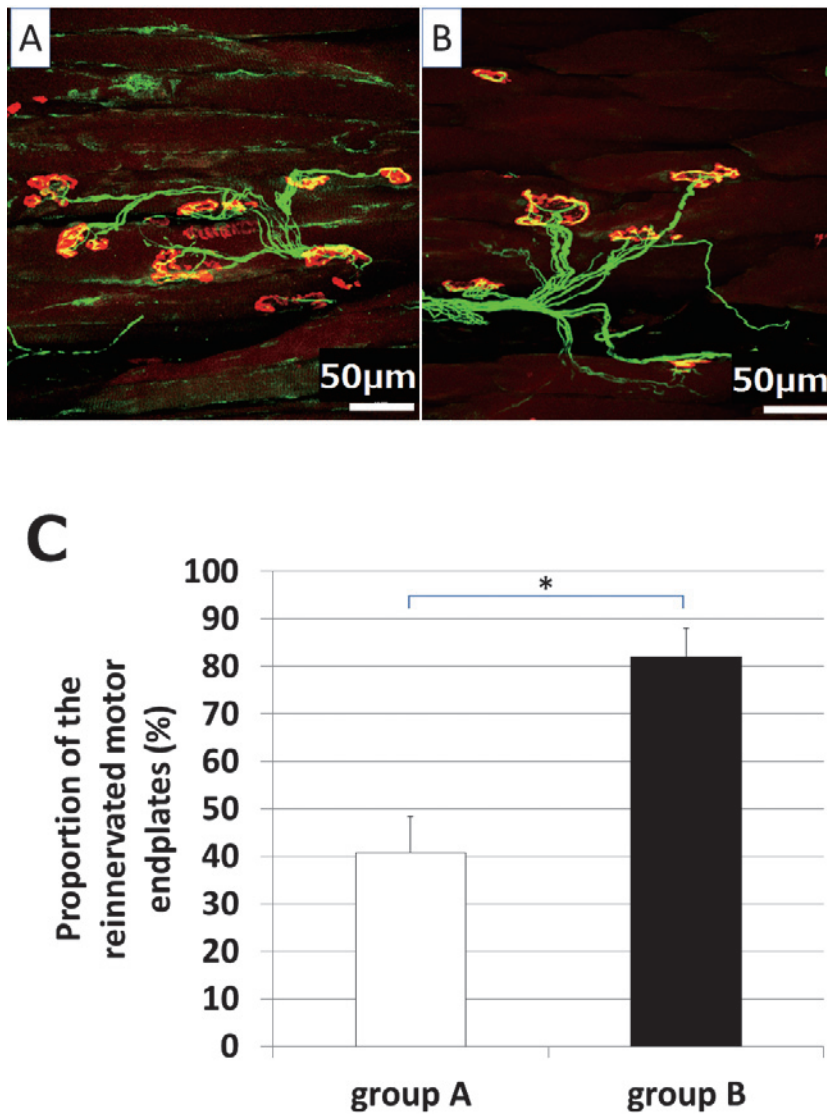


Fig. 4 Formation of neuromuscular junctions in the tibialis anterior muscle
 Formation of neuromuscular junctions in the tibialis anterior muscle was evaluated in group A (A) and group B (B). Green = pan-neuronal marker; red = α -bungarotoxin. Group B had a greater proportion of reinnervated motor endplates (C). Bar graphs represent mean \pm standard error * $p < 0.05$.

the regeneration of neuronal axons. Thus, the optimal timing of transplantation is thought to be 1 to 2 weeks after injury.⁵⁾ However, treating peripheral nerve injury in the clinical setting, several investigators have stated that reconstruction of denervated muscle using nerve transfer techniques within 9 months of injury notably improves functional outcome.^{19, 20)} Considering this, peripheral nerve reconstruction can be performed with MISM even if the treatment is delayed up to 6 months after injury.

The MISM technique has several shortcomings. The reinnervated muscle cannot be voluntarily

contracted, since MISM is isolated from the central nervous system. However, recent developments in brain-machine interfaces may enable translation of neural signals from the brain into control signals for guiding MISM.²¹⁾ We are also investigating if MISM can be controlled by feedback signals provided by an electromyogram or an external sensor, such as an accelerometer or a sensory prosthetic. This local feedback could restore the function of denervated muscles disconnected from the central nervous system. Another shortcoming is the source of transplanted cells. In the present experiments, we implanted embryonic ventral spinal cord MNs. However, the use of embryonic MNs for cell therapy is ethically problematic in clinical settings. Theoretically, MNs derived from embryonic stem (ES) cells or induced pluripotent stem cells can be alternatives. Yohn *et al.* have already succeeded in transplanting ES cell-derived MNs in the peripheral nerves of mice.²²⁾ Therefore, MISM can be generated with these alternative cell sources.

CONCLUSION

This study demonstrates the optimum number of MNs required to restore muscle function. When sufficient MNs are transplanted into the peripheral nerve, muscle atrophy is reduced and denervated muscles are restored. Recent advances in stem cell research and biomedical engineering have opened up possibilities for the clinical application of MISM. MISM with FES provides a new treatment strategy for paralyzed muscles.

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