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Bioabsorbable nerve conduits coated with induced pluripotent stem cell-derived

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Takuya Yokoi [1], Takuya Uemura [1]*, Kiyohito Takamatsu [2], Kosuke Shintani [1], Ema Onode [1],

Mitsuhiro Okada [1], Noriaki Hidaka [3], Hiroaki Nakamura [1]

[1]Department of Orthopaedic Surgery, Osaka City University Graduate School of Medicine,

Osaka, Japan

[2]Department of Orthopaedic Surgery, Yodogawa Christian Hospital, Osaka, Japan

[3]Department of Orthopaedic Surgery, Osaka City General Hospital, Osaka, Japan

*Corresponding author: Takuya Uemura, MD, PhD, Department of Orthopaedic Surgery,

Osaka City University Graduate School of Medicine, 1-4-3 Asahimachi Abeno-ku, Osaka

545-8585, Japan. Tel: +81-6-6645-3851. Fax: +81-6-6646-6260.

E-mail: t-uemura@med.osaka-cu.ac.jp

Abstract

Aging influences peripheral nerve regeneration. Nevertheless, most basic research of bioabsorbable nerve conduits including commercial products have been performed in very young animals. Results from these studies may not provide information about axonal regeneration in aged tissue, because young nerve tissue holds sufficient endogenous potential for axonal regeneration. The clinical target age for nerve conduit application is most likely going to increase with a rapidly growing elderly population. In the present study, we examined axonal regeneration after sciatic nerve defects in aged and young mice. Five-millimeter sciatic nerve defects in young (6 weeks old) and aged (94 weeks old) mice were reconstructed using nerve conduits (composed of a poly lactide and caprolactone) or autografts. In addition, in aged mice, sciatic nerve defects were reconstructed using nerve conduits coated with mouse induced pluripotent stem cell (iPSc)-derived neurospheres. Using electrophysiological and histological techniques, we demonstrated axonal regeneration was significantly less effective in aged than in young mice both for nerve conduits and for nerve autografts. However, despite the low regenerative capacity of the peripheral nerve in aged mice, axonal regeneration significantly increased when nerve conduits coated with iPSc-derived neurospheres, rather than nerve conduits alone, were used. The present study shows that aging negatively affects peripheral nerve regeneration based on nerve conduits in mice. However, axonal regeneration using nerve conduits was improved when supportive iPSc-derived neurospheres were added in the aged mice. We propose that tissue-engineered bioabsorbable nerve conduits in combination with iPSc-derived neurospheres hold therapeutic potential both in young and elderly patients.

Introduction

Currently, nerve autografting is the gold standard in clinical settings to salvage peripheral nerve defects. However, because a functional normal nerve needs to be sacrificed, autograft harvesting has severe disadvantages such as donor-site morbidity and limited supply.[1] Various artificial bioabsorbable nerve conduits have been developed as an alternative to nerve autografts to repair peripheral nerve injuries.[1-3] Some of these nerve conduits are starting to be clinically available, because results from basic animal research were satisfactory.[4-6] However, sufficient axonal regeneration using nerve conduits for peripheral nerve defects have been observed only in very young animals (6 to 10 week-old rodents).[7-9] The age of these animals is equivalent to about three years of age in humans.[10,11] Whereas, young tissue has high peripheral regenerative capacity, in aged tissue the potential for nerve regeneration and functional recovery after injury decreases.[12-15] Very few studies of nerve conduits have focused on peripheral nerve regeneration in aged animal models.[16] Therefore, more studies are needed to explore the applicability of nerve conduits for axonal regeneration in aged animals to estimate the therapeutic potential of nerve conduits for the growing aging population.

We have previously reported that induced pluripotent stem cell (iPSc)-derived neurospheres as supportive cells for nerve conduits accelerated nerve regeneration in sciatic nerve defects in young mice.[17-19]

In the present study, we first examined peripheral nerve regenerative capacity both for nerve conduits and nerve autografts in aged mice compared with young mice. Subsequently, we examined whether addition of iPSc-derived neurospheres to the nerve conduit positively

affected peripheral nerve regeneration in sciatic nerve defects in aged mice.

Materials and Methods

First Experiment: Peripheral nerve regeneration using nerve autografts and conduits in sciatic nerve defects in aged and young mice

Experimental Groups

Six-week-old (young) and 92-week-old (aged) male mice (C57BL/6) were used in the present study. Mice were purchased from Japan SLC (Hamamatsu, Japan) and housed in an air-conditioned room with free access to food and water. A total of 58 mice (comprising 24 young mice and 34 aged mice) were divided into the following four experimental groups:

(1) Young mice treated with autogenous nerve grafts (young autograft group, 14 mice)

(2) Aged mice treated with autogenous nerve grafts (aged autograft group, 17 mice)

(3) Young mice treated with nerve conduits (young conduit group, 10 mice),

(4) Aged mice treated with nerve conduits (aged conduit group, 17 mice).

Experiments were conducted in strict accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals of Osaka City University.

Nerve Conduit

Previous studies have also used bioabsorbable polymer tubes (outer diameter: 2 mm, inner diameter: 1 mm, length: 7 mm) as artificial nerve conduits in the present study.[17-19] The polymer tube consists of two layers: (1) an outer layer composed of a poly L-lactide multifilament fiber mesh, and (2) an inner layer composed of a 50% poly L-lactide and 50% poly caprolactone porous sponge. This sponge possesses high flexibility and absorbability. The nerve conduit was elastic enough to maintain its tubular structure during

axonal growth but flexible enough to allow easy handling.[17-19] The nerve conduits were pre-wet with a 70% ethanol solution and then rinsed with physiological saline. The ethanol solution allowed wetting of the hydrophobic polymer by aqueous-based solutions and suspension.[20]

Surgical Procedure

Complete 5-mm defects were induced in the left sciatic nerve and repaired using nerve autografts or nerve conduits (Figure 1). In the autograft group, nerve defects were reconstructed using the resected nerve itself. The nerve was rotated 180º, inserted between the two nerve stumps, and sutured under the microscope with 9-0 nylon sutures. In the nerve conduit group, a bioabsorbable polymer tube was inserted between the proximal and distal nerve stumps. Briefly, 1-mm pieces of both proximal and distal ends of the nerve were pulled into the nerve conduit and nerve ends were sutured at two locations to the lumen wall with 9-0 nylon sutures under a microscope.[17-19] Twelve weeks after repair of the peripheral nerve gaps, axonal regeneration was assessed electrophysiologically and histologically as described below.

Second Experiment: Peripheral nerve regeneration after sciatic nerve defect using nerve conduits alone and nerve conduits coated with iPSc-derived neurospheres in aged mice

Experimental Groups

A total of 32 aged mice were randomly separated into two experimental groups. The first group was treated with nerve conduits alone (aged conduit group, 17 mice). The second

group was treated with nerve conduits coated with iPSc-derived secondary neurospheres (aged iPS group, 15 mice). Nerve couduits were implanted using the same procedure as described earlier. Twelve weeks after repair of the peripheral nerve gaps, axonal regeneration was assessed electrophysiologically and histologically as described below.

Cell Culture and Neural Induction of iPScs

Mouse iPSc from the iPS-MEF-Ng-178B-5 cell line was provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan.[21] For neural induction, we generated neurospheres containing neural stem/progenitor cells from the iPScs, following a published method.[22]

iPScs grown on gelatin-coated (0.1%) tissue culture dishes were maintained in standard embryonic stem cell medium (Gibco, Tokyo, Japan), then dissociated, and cultured in suspension as embryoid bodies (EBs), which contain progenitor cells of the three germ layers. To enrich for neural stem/progenitor cells in the EBs, a low concentration of retinoic acid (1.0×10^{-8} M) was added during EB formation. We dissociated the EBs and selectively expanded the neural stem/progenitor cells in serum-free medium containing fibroblast growth factor (FGF)-2 (Wako, Osaka, Japan) as neurospheres in the following manner. To form primary neurospheres, day6 EBs were dissociated and cultured in suspension at 5.0×10^{4} cells/ml for 7days in serum-free Dulbecco's modified Eagle's medium (DMEM) supplemented with N-2 and B-27 supplement (Gibco, Tokyo, Japan) and 20 ng/ml FGF-2. Day 4 primary neurospheres were transferred from a cell culture flask (Nunc, Yokohama, Japan) to an ultra-low attachment dish (Corning, Tewksbury, Mass., USA). To form

secondary neurospheres, day7 primary neurospheres were dissociated with TrypLESelect (Gibco) and cultured in the same culture medium as primary neurospheres. Day 4 secondary neurospheres were transferred from a cell culture flask to an ultra-low attachment dish. Then, day 7 secondary neurospheres were used for coating the nerve conduits.

Seeding Secondary Neurospheres on the Conduit

Nerve conduits coated with iPSc-derived secondary neurospheres were prepared following the same procedure as previously described.[17-19] Briefly, nerve conduits were moistened with 70% ethanol and rinsed with physiological saline solution. Next, day-7 secondary neurospheres were carefully suspended over the nerve conduits at a density of 1,500,000 cells per conduit. Finally, nerve conduits were transferred to Dulbecco's modified Eagle's medium supplemented with 10% embryonic stem cell- qualified fetal bovine serum (all from Gibco Life Technologies, California, USA) and incubated for 14 days.[23] This completed the process of coating the nerve conduits with iPSc-derived secondary neurospheres and more than half of the coating cells were differentiated into Schwann-like cells that were important for axonal regeneration in nerve conduit scaffolds as described in detail previously.[19]

Evaluations for the First and Second Experiments

Electrophysiological Analysis

The recovery of motor function of each mouse's hind limb was assessed on the basis of

electrophysiological recordings from the sciatic nerve as has been described previously.[24] Briefly, mice were anesthetized and bilateral sciatic nerves were carefully exposed. Electrophysiological signals were obtained using a Viking Select Nicolet® (Natus Neurology Inc., Wisconsin, US) electromyogram machine. The nerve at the proximal end of the nerve conduit was stimulated with a monopolar 28G needle electrode both in conduit and iPS groups. The nerve proximal to the suture point was stimulated with a monopolar 28G needle electrode in the autograft group. The recording needle electrode was placed at the gastrocnemius muscle. The reference needle electrode was placed at achilles tendon. A series of nerve stimulations was performed with repetitively generated single pulses of 0.1 ms duration until a maximal artifact-free compound muscle action potential (CMAP) motor response was evoked. CMAP amplitudes were recorded at three different recording locations to optimize recording conditions. CMAP amplitude ratios of experimental to unaffected side are reported.

Histological Evaluation and Histomorphometry

Twelve weeks after surgery, nerve conduits and grafted nerves were harvested, fixed in 4% paraformaldehyde overnight and embedded in paraffin. Axonal regeneration was examined immunohistochemically in central transverse sections using anti-neurofilament protein antibodies (DAKO, California, United States). For each nerve conduit and grafted nerve, the section showing the largest number of regenerating axons was photographed at 400× magnification with an Olympus DP70 camera (Olympus, Tokyo, Japan).[17-19] Images were analyzed using software ImageJ (National Institute of Health, http://imagej.nih.gov/ij/) and

regenerating axons positive for neurofilament protein were counted automatically using a command of analyze particles.

Data Processing and Statistical Analysis

All statistical analysis was performed using Prism 6.0 (GraphPad Software, USA). Data are reported as mean ± standard deviation (SD). Mann-Whitney tests were used to compare between groups. Differences were considered statistically significant at $p<0.05$.

Results

Peripheral Nerve Regeneration using Nerve Grafts and Conduits in Aged and Young Mice with Sciatic Nerve Defects

In both young and aged mice, autogenous nerve grafts did not dissociate from the nerve stumps and the luminal structure of the nerve conduits remained stable without collapse 12 weeks after implantation (Figure 1A-D). In the autograft group, CMAP amplitudes were significantly smaller in aged than young mice (Figure 2A). Similarly, in the nerve conduit group, CMAP amplitudes were significantly smaller in aged than young mice (Figure 2B). Figures 2C-F show histological analyses of regenerative axons positive for anti-neurofilament protein antibody in nerve autografts and conduits in young and aged mice. In the autograft group, the number of neurofilament-positive axons was significantly lower in aged than in young mice as shown in the morphometric analysis (p<0.001; Figure 2G). In the nerve conduit group, neurofilament-positive axons tended to be fewer in aged than in young mice. However this tendency was not statistically significant (p = 0.64; Figure 2H). Taken together, these results indicate that the capacity for peripheral nerve regeneration using nerve autografts and conduits decreases with age.

Peripheral Nerve Regeneration using Nerve Conduits Alone and Nerve Conduits Coated with iPSc-derived Neurospheres in Aged Mice with Sciatic Nerve Defects

CMAP amplitudes were significantly higher in aged mice treated with iPSc-derived neurospheres in addition to nerve conduits than in aged mice treated with nerve conduit alone (Figure 3A). Regenerative axons were apparent in histological analyses, both in aged

mice treated with nerve conduits coated with iPSc-derived neurospheres and with nerve conduits alone (Figure 3B-E). However, the number of neurofilament-positive axons was significantly larger in aged iPS group than in the aged conduit group as shown by morphometric analysis (Figure 3F). Taken together, these results indicate that the addition of iPSc-derived neurospherers to nerve conduits enhanced axonal regeneration in aged mice, although age mice showed less regenerative capacity than young mice.

Discussion

In the present study, we show that aging negatively affects axonal regeneration both using nerve grafts and nerve conduits in mice with sciatic nerve defects. However, peripheral nerve regeneration with nerve conduits could be enhanced by the addition of iPSc-derived neurospheres in aged mice. To our knowledge, the present study is the first to demonstrate the effect of supportive cells such as iPSc to nerve conduits on peripheral nerve regeneration in aged animals with decreased regenerative capacity.

The aging world population is progressively growing and is therefore the target population for nerve autografts and conduits applications. Several clinical investigations have reported that the rate and the degree of recovery after peripheral nerve injuries decline with age.[14,25] A number of basic studies have investigated effects of aging and aging-related delay mechanism on peripheral nerve regeneration using nerve crush or repair models for nerve transection in aged animals.[26-29] However, very few studies have investigated peripheral nerve regeneration using nerve autografts or nerve conduits for peripheral nerve gaps in aged animals. In contrast, basic research on peripheral nerve regeneration using nerve conduits in young animals is abundant, despite the fact that young animals might have sufficient endogenous potential for peripheral nerve regeneration.[8,9,30] Similarly, satisfactory axonal regeneration and functional outcomes in peripheral nerve defects using nerve conduits have been reported only in young animal.[8,9,18,30] However, the intrinsic capacity of nerve regeneration in young animals may contribute significantly to axonal growth in nerve conduits. Therefore, the effect of the addition of supportive cells to nerve conduits may have been overestimated in young animal models. We believe that it is

necessary to precisely evaluate the feasibility of nerve conduits and the effect of supportive cells on nerve conduits not only in young but also in aged animal models to close the discrepancy between basic research and clinical practice.

Clavijo-Alvarez et al. used biodegradable nerve conduits to treat sciatic nerve defects in aged rats (11 months old).[16] No improvement in functional recovery was observed in aged rats, independent of the type of nerve conduits employed. However, Clavijo-Alvarez et al. did not relate their findings to functional recovery in young rats. In contrast, the comparison of axonal regeneration using nerve conduits between young and aged mice is one of the strengths of the present study. Scheib et al. histologically evaluated sciatic nerve grafting in young (2 months old) and aged rats (18 months old).[31] The number of myelinated regenerative axons in the nerve grafts was lower in aged than in young rats owing to insufficient clearance of debris by macrophages and Schwann cells in aged animals. Our data support these results and suggest additionally that electrophysiological recovery is impaired in aged mice. Thus, recent studies on the influence of aging on peripheral nerve regeneration have shown that Wallerian degeneration and remyelination of axons are delayed in aged animals, because recruitment and response of Schwann cells and macrophages to axonal and myelin debris are diminished.[31] Furthermore, levels of neurotrophic factors secreted by Schwann cells have been shown to be lower in aged animals.[32]

The degradation process of the nerve conduits used in the present study has already been demonstrated in detail.[19] The material comprising the nerve conduits was gradually absorbed without collapse and was almost totally absent 48 weeks after implantation in

mice. This long time-span of degradation is extremely advantageous for axonal regeneration, as previously reported by Shin *et al.*[33] In their study comparing commercially available biodegradable synthetic nerve conduits, the poly-lactide-caprolactone conduits, which were composed of material similar to our nerve conduit, remained structurally stable without collapse at 12 weeks after implantation in rats, owing to their slow degradation rate. In contrast, the soft polyglycolic acid conduits completely collapsed, because they were absorbed faster. As collapse of the conduits interfered with axonal regeneration, the polyglycolic acid conduits had the poorest functional recovery, while the poly-DL-lactide-e-caprolactone conduits were similar to autografts with respect to functional recovery.

In the present study, we provided first evidence of increased axonal regeneration after addition of iPSc-derived neurospheres to nerve conduits in aged mice. The iPSc-derived secondary neurospheres after 14 days of nerve conduit culture comprised mainly of glial cells.[19] According to our previous research on the multipotential characteristics of iPSc-derived secondary neurospheres, about three quarters differentiated into the immature Schwann-like cells (characterized by immunohistochemistry with anti-GFAP and anti-S-100 antibodies), while a quarter of them were neurons.[19] Here, we used the term Schwann-like because it has not yet been proven that iPSc-derived Schwann-like cells possess the same properties and specific RNA expression in vitro as native Schwann cells. As mentioned above, recruitment and response of Schwann cells and macrophages to axonal and myelin debris during Wallerian degeneration decrease with age.[32] With regard to the potential mechanism of action of transplanted cells, grafted iPSc-derived immature Schwann-like cells could be indirectly responsible as supportive cells for the release of

some kinds of growth factors, probably resulting in enhanced recruitment of endogenous Schwann cells and macrophages and clearance of myelin debris, or direct reformation of the myelin sheath. Further, the effects of grafted cells on aged animals might be enriched following transplantation of younger cells, as in the present study. Further studies are necessary to elucidate the exact mechanisms by which iPSc-derived Schwann-like cells promote the regeneration of peripheral nerves.

Conclusions

We show that axonal regeneration after sciatic nerve defects was less effective in aged than in young mice when using either nerve conduits or nerve autografts. The addition of iPSc-derived neurospheres to nerve conduits enhanced axonal regeneration in aged mice, despite the fact that regenerative capacity of peripheral nerves was low. Our results suggest that tissue-engineered bioabsorbable nerve conduits in combination with iPSc-derived neurospheres hold therapeutic potential for peripheral nerve defects both in young and aged patients.
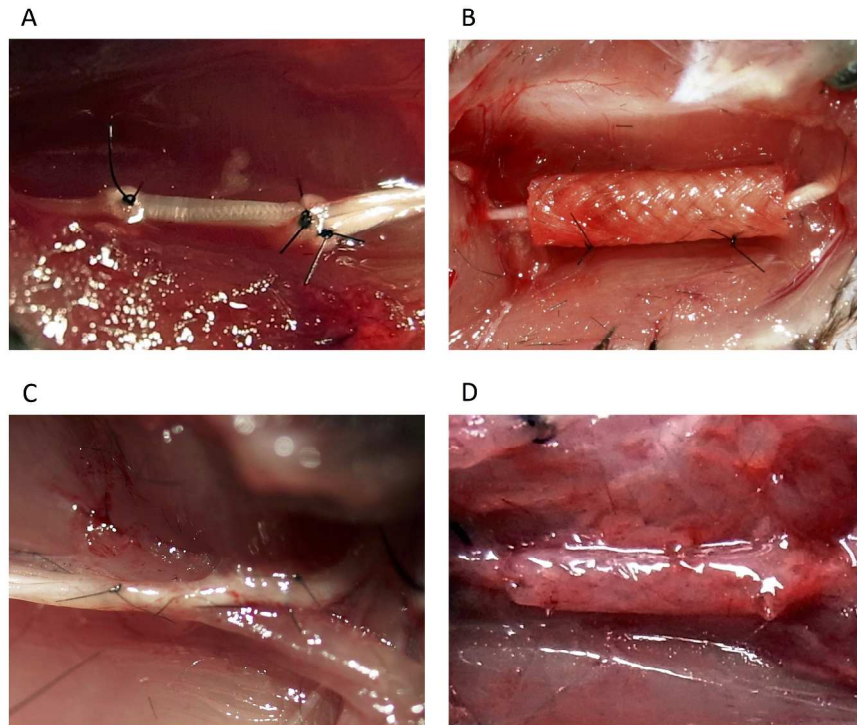
Figure Legends

Figure 1. Peripheral nerve reconstruction after sciatic nerve defects using autogenous nerve grafts (A) and nerve conduit (B) at the time of surgery, and autogenous nerve graft (C) and nerve conduit (D) 12 weeks after surgery.

Figure 1

Figure 1. Peripheral nerve reconstruction after sciatic nerve defects using autogenous nerve grafts (A) and nerve conduit (B) at the time of surgery, and autogenous nerve graft (C) and nerve conduit (D) 12 weeks after surgery.

254x338mm (300 x 300 DPI)

Figure 2. Electrophysiological recovery of sciatic nerve in autograft groups (A) and conduit groups (B) 12 weeks after surgery. Immunohistochemistry of anti neurofilament protein antibody in central transverse sections in the young autograft group (C), the aged autograft group (D), the young conduit group (E), and the aged conduit group (F) 12 weeks after surgery. Scale bars, 50 μm. Quantitative analysis of immunohistochemistry in autograft groups (G) and in conduit groups (H). Bars and lines represent mean ± SD (A, B, G, and H).
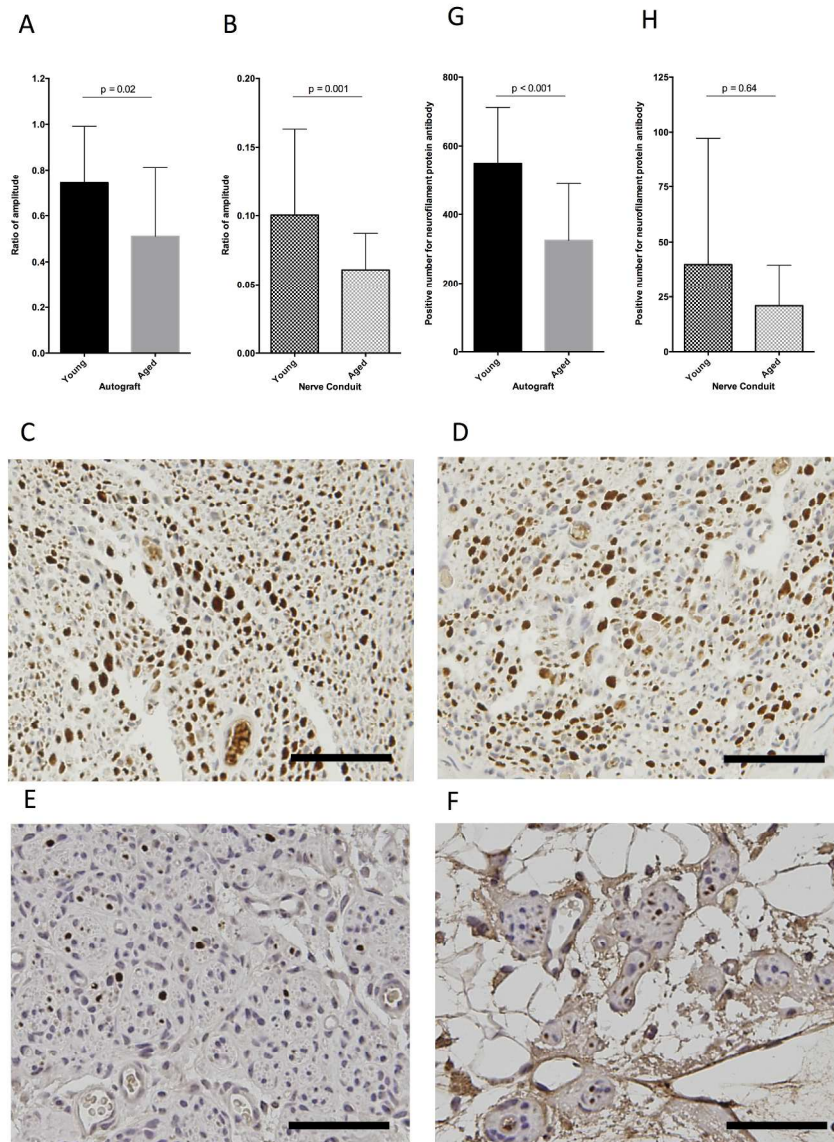
Figure 2A,2B,2C,2D,2E,2F,2G,2H

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254x338mm (300 x 300 DPI)

Figure 3. (A) Electrophysiological recovery for sciatic nerve defects treated with nerve conduits coated with iPSc-derived neurospheres and nerve conduits alone in aged mice 12 weeks after implantation. Central transverse sections of the nerve conduits from the aged conduit group (B and D) and from the aged iPSc group (C and E) 12 weeks after implantation. Hematoxylin and eosin staining (B and C). Scale bar, 50 mm (B and C). Immunohistochemical staining of neurofilament protein (D and E). Regions marked with rectangles in D and E are depicted at a higher magnification in B and C. Scale bar, 200 mm (D and E). (F) Quantitative analysis of the number of neurofilament-positive axons. Bars and lines represent mean ± SD (A and F).
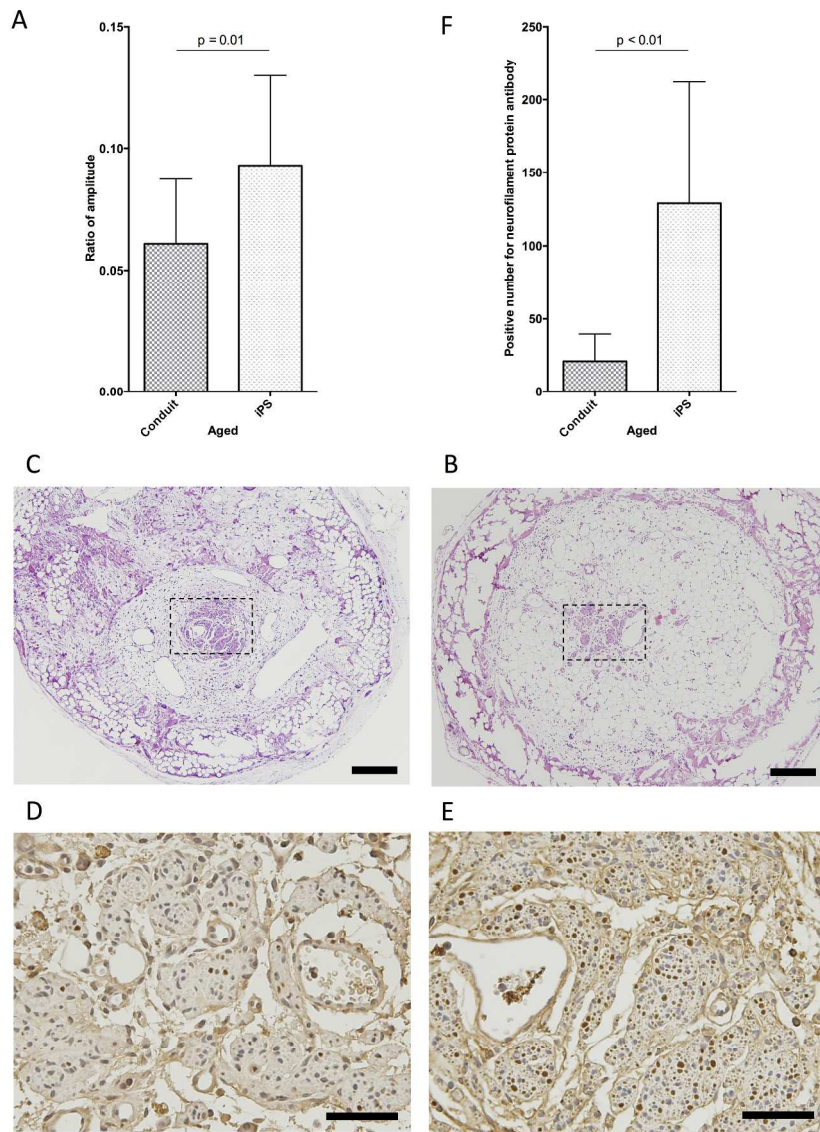
Figure 3

Figure 3. (A) Electrophysiological recovery for sciatic nerve defects treated with nerve conduits coated with iPSc-derived neurospheres and nerve conduits alone in aged mice 12 weeks after implantation. Central transverse sections of the nerve conduits from the aged conduit group (B and D) and from the aged iPSc group (C and E) 12 weeks after implantation. Hematoxylin and eosin staining (B and C). Scale bar, 50 mm (B and C). Immunohistochemical staining of neurofilament protein (D and E). Regions marked with rectangles in D and E are depicted at a higher magnification in B and C. Scale bar, 200 mm (D and E). (F) Quantitative analysis of the number of neurofilament-positive axons. Bars and lines represent mean ± SD (A and F).

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Conflict of Interest

The authors declare no conflict of interest.

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