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Transplantation of induced pluripotent stem cell-derived neurospheres for peripheral nerve repair

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ABSTRACT

In spite of the extensive research using induced pluripotent stem (iPS) cells, the therapeutic potential of iPS cells in the treatment of peripheral nerve injury is largely unknown. In this study, we repaired peripheral nerve gaps in mice using tissue-engineered bioabsorbable nerve conduits coated with iPS cell-derived neurospheres. The secondary neurospheres derived from mouse iPS cells were suspended in each conduit (4000,000 cells per conduit) and cultured in the conduit in three-dimensional (3D) culture for 14 days. We then implanted them in the mouse sciatic nerve gaps (5 mm) (iPS group; $n = 10$). The nerve conduit alone was implanted in the control group ($n = 10$). After 4, 8 and 12 weeks, motor and sensory functional recovery in mice were significantly better in the iPS group. At 12 weeks, all the nerve conduits remained structurally stable without any collapse and histological analysis indicated axonal regeneration in the nerve conduits of both groups. However, the iPS group showed significantly more vigorous axonal regeneration. The bioabsorbable nerve conduits created by 3D-culture of iPS cell-derived neurospheres promoted regeneration of peripheral nerves and functional recovery in vivo. The combination of iPS cell technology and bioabsorbable nerve conduits shows potential as a future tool for the treatment of peripheral nerve defects.

1. Introduction

Recently, there have been great advancements in the study of induced pluripotent stem cells (iPS) cells in the field of regenerative medicine [1,2]. The iPS cells have the ability to differentiate into various types of somatic cells, such as cardiomyocytes, hepatic cells, and pancreatic cells, and thus have been used for understanding the mechanisms of diseases, development of new drugs, and for regenerative therapy including cell implantation [3–7]. Recently, methods for neural induction of iPS cells have been established by Okada and Miura [8,9]. In these studies, iPS cells were differentiated into neural precursor cell aggregates, so called neurospheres, which are then able to differentiate into neurons and glial cells. By application of the same method of neural induction of iPS cells for the regenerative therapy of the central nervous system, Nakamura et al. confirmed that grafted human-iPS-cell-derived neurospheres promoted motor functional recovery after spinal cord injury in mice [10–12]. However, there have been few reports of the application of iPS cells for regenerative therapy of peripheral nerves. The purpose of this study was to repair sciatic nerve gaps in mice using bioabsorbable nerve conduits coated with iPS cell-derived neurospheres, utilizing the neural induction of iPS cells for peripheral nerve regeneration.

2. Materials and methods

2.1. Neural induction of iPS cells

We used mouse iPS cells of the iPS-MEF-Ng-178B-5 cell line that was established using three transcription factors, Oct3/4, Sox2 and Klf4. iPS cells were provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan [13]. iPS cells were cultured as previously described [1,2]. For neural induction, we generated neurospheres containing neural stem/progenitor cells from the iPS cells using a published method [8,9]. After embryoid body formation, iPS cells formed primary neurospheres in the presence of fibroblast growth factor 2. Primary neurospheres were then dissociated to form secondary neurospheres. The primary neurospheres are known to differentiate mainly into neurons, while

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the secondary neurospheres are known to differentiate into mainly glial lineage cells, in addition to neurons [14].

2.2. Nerve conduits

We used a bioabsorbable polymer tube (outer diameter: 2 mm, inner diameter: 1 mm, length: 7 mm) that we developed and have been reporting on for an artificial nerve conduit for the treatment of peripheral nerve defects [15] (Fig. 1A). The lumen wall of the tube was two-layered. The inner layer was made of sponge copolymer composed of a 50% poly L-lactide (PLA) and 50% poly ε-caprolactone (PCL), which possess high flexibility and absorbability. The outer layer was made of PLA multifilament fiber mesh, which reinforces the tube because the inner layer alone does not provide enough strength to the tube. This two-layered structure not only prevents the lumen from collapsing after transplantation, it also provides enough flexibility to the tube (Fig. 1B). In particular, the PLA and PCL copolymer sponge of the inner layer was a honeycomb structure with pores of 10–50 μm, into which regeneration–facilitating cells such as Schwann cells could enter (Fig. 1C).

2.3. Preparation of nerve conduits coated with neurospheres

The conduits were pre-wet with a 70% ethanol solution and then rinsed with physiologic saline. The ethanol solution allowed wetting of the hydrophobic polymer by aqueous-based solutions and suspensions [15]. The secondary neurospheres were dissociated into single cells using TripleLE select (Invitrogen, Osaka, Japan) and were carefully suspended over each conduit at a density of 4000,000 cells per conduit. After suspension, these conduits were placed in Dulbecco's modified Eagle's medium supplemented with 10% embryonic stem (ES) cell-qualified fetal bovine serum (Invitrogen) for 14 days. Before implantation of the nerve conduits, they were stained with hematoxylin-eosin (HE) at the central transverse section in order to confirm the adhesion of neurospheres to their inner layer.

2.4. Repairing sciatic nerve gaps of mice

The experimental group (iPS group) is a group of ten 6-year-old mice (C57BL6), whose complete 5-mm defects in the left sciatic nerve were reconstructed by the nerve conduits coated with iPS cell-derived secondary neurospheres. The control group is a group of ten mice, whose 5-mm sciatic nerve gaps were reconstructed by the nerve conduit alone. Both proximal and distal ends of the nerve were pulled into the nerve conduit by a length of 1 mm, and the nerves were sutured together with 9–0 nylon suture at two locations on the lumen wall of the inner layer (Fig. 2A).

2.5. The evaluation of functional recovery

The recovery of motor and sensory function of the mouse's hindlimb was assessed at 4, 8 and 12 weeks after repair of the peripheral nerve gaps with nerve conduits. The recovery of motor function was assessed by walking track analysis as described previously [16,17]. Measurements of print length were made on the experimental side (experimental print length; EPL) and the normal side (normal print length; NPL). The print length factor (PLF) was then calculated from a mean of four values for the EPL and NPL as follows: PLF = (EPL-NPL)/NPL. The recovery of sensory function was assessed by the hot water behavior test as described previously [18,19]. Mice were immobilized by hands and the hindlimbs of the experimental and normal side, respectively, were submerged into the water bath (50 °C). The average foot withdrawal time from hot water stimulus was evaluated. Each hindlimb was tested four times, alternating sides, with a 30-s interval between tests.

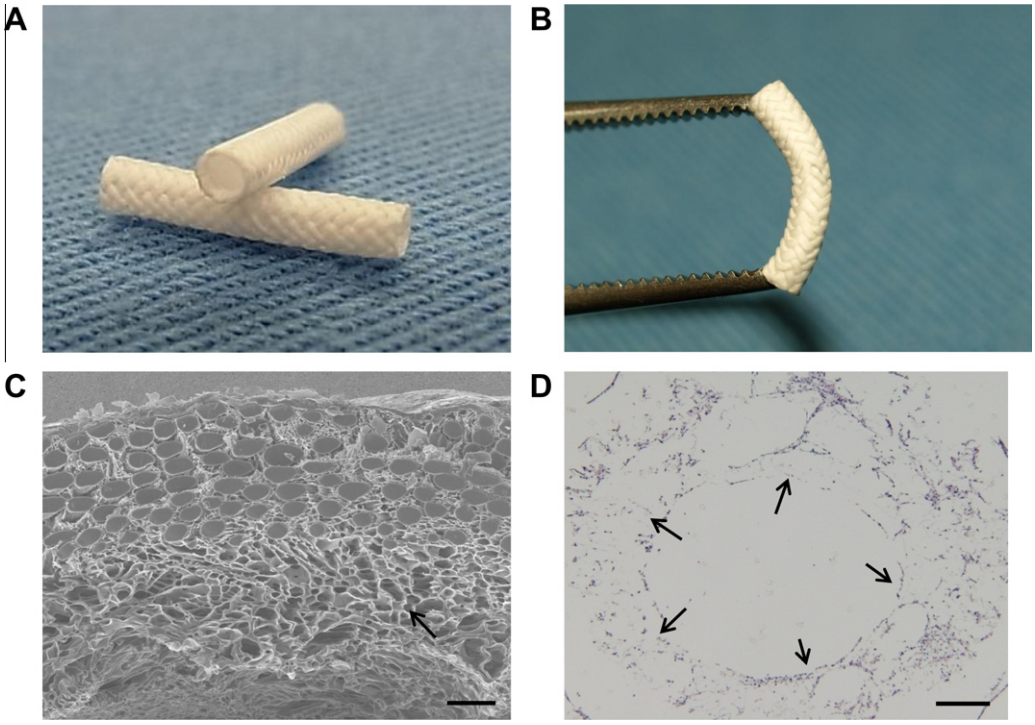

Fig. 1. The bioabsorbable nerve conduits. (A and B) Gross appearance. The conduit was elastic enough to maintain its tubular structure but flexible enough to allow easy handling. (C) Scanning electron microscope (SEM) image of a cross-section of the two-layered conduit. The inner layer (arrow) was honeycomb structure containing pores of 10–50 μm into which cells can enter. *Scale bar* 50 μm. (D) The hematoxylin-eosin image of cross sectioned nerve conduit coated with iPS cell-derived secondary neurospheres. The iPS cell-derived secondary neurospheres were adhered to the inner surface of the nerve conduits (arrow) and were migrated into the inner porous sponge. *Scale bar* 200 μm.

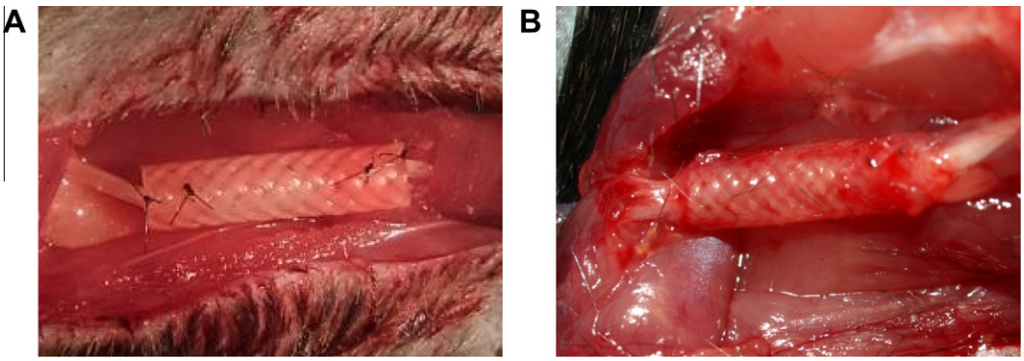

Fig. 2. Reconstructed sciatic nerve gap with the nerve conduit. (A) At the time of the implantation. (B) 12 weeks after implantation.

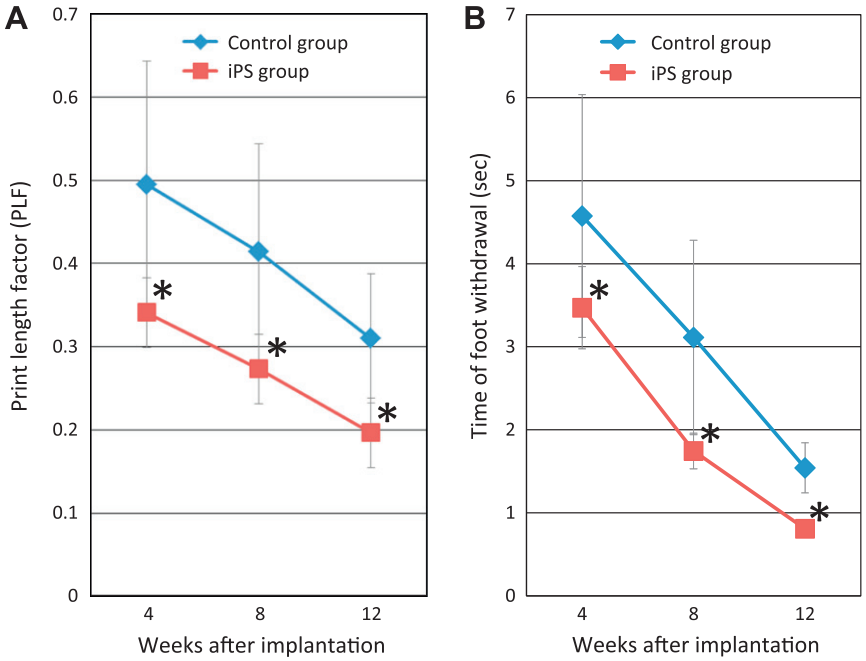

Fig. 3. The recovery of motor and sensory function. (A) The recovery of motor function assessed by the print length factor (PLF). (B) The recovery of sensory function assessed by the time of foot withdrawal reflex. Bars and lines represent mean ± SD. $^{*}p < 0.05$.

2.6. Histological evaluation and histomorphometry

At 12 weeks, the nerve conduits were harvested and nerve regeneration was examined by immunohistochemistry with the following primary antibodies: anti-βIII tubulin (Tuj1, DAKO, Tokyo, Japan), a marker of primitive neurons; anti-neurofilament protein antibodies (DAKO), a marker of mature neurons; anti-glial fibrillary acidic protein (GFAP, DAKO), a marker of early Schwann cells; and anti-S-100 (DAKO), a marker of Schwann cells at the central transverse section of nerve conduits. For each nerve conduit, an image in which the greatest regenerative nerves were found was photographed at a magnification of 200× with a Olympus DP70 camera (Olympus, Tokyo, Japan) and analyzed using morphometry software, Winroof (Mitani, Tokyo, Japan). Areas that were positive for the neurofilament protein antibody and S-100 antibody were automatically counted.

2.7. Statistical analysis

Data were expressed as the means ± standard deviation (SD). Differences between the groups were assessed using the Mann–Whitney test. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. The nerve conduits coated with iPS cell-derived neurospheres

The iPS cell-derived secondary neurospheres were adhered to the inner surface of the nerve conduits and had migrated into the inner porous sponge in the HE-stained images (Fig. 1D). The iPS cell-derived neurospheres could be grafted onto the nerve conduits and three-dimensional (3D)-cultured in the nerve conduits as a scaffold.

3.2. Functional analysis

The recovery of motor function was assessed by walking track analysis. The mean values of the PLF in the iPS group were significantly lower than in the control group at 4, 8 and 12 weeks, respectively (Fig. 3A). The recovery of sensory function was assessed by the hot water behavior test. The average time of foot

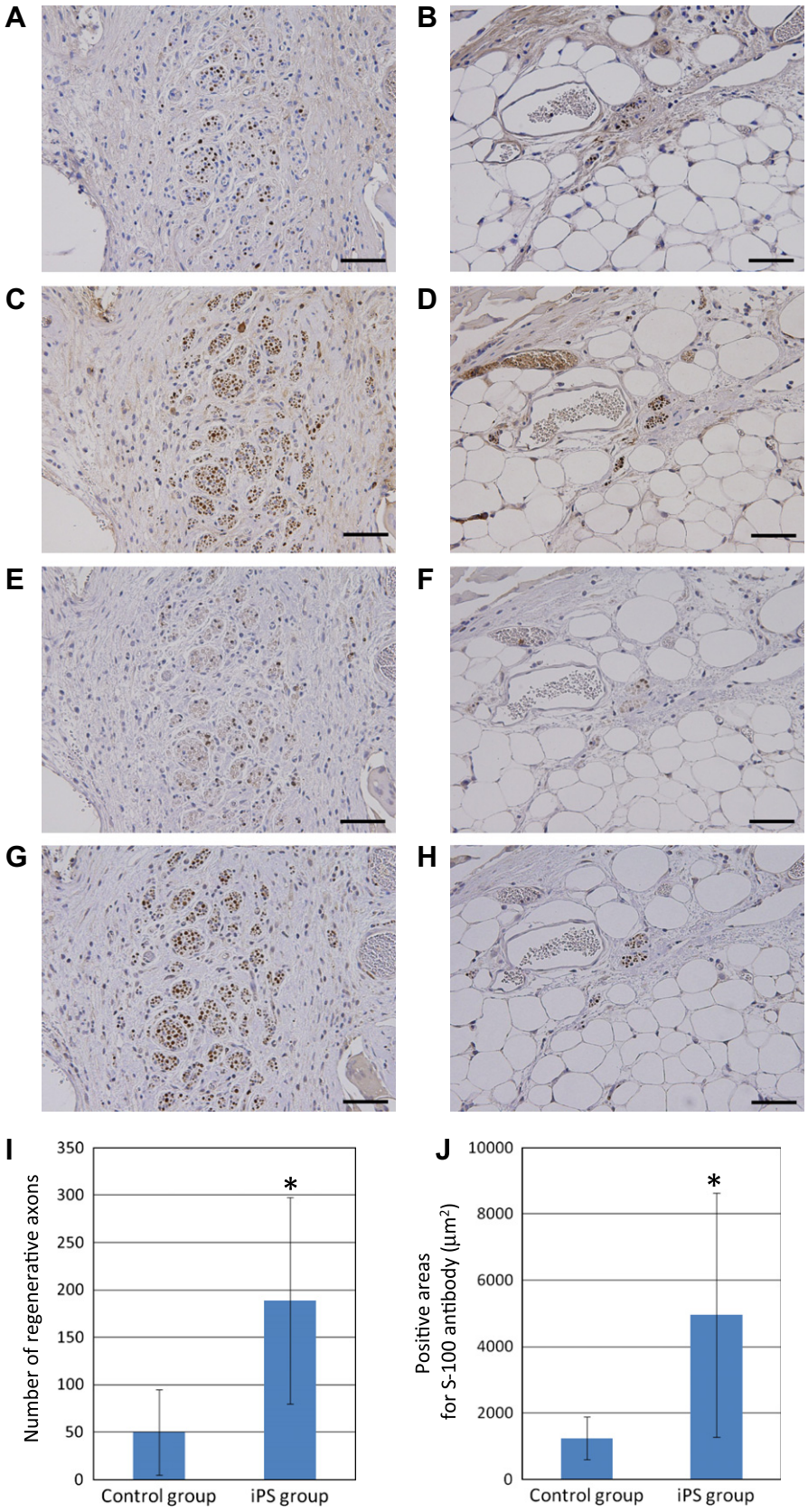

Fig. 4. The central transverse sections of the nerve conduits at 12 weeks after implantation. (A and B) Immunohistochemistry for neurofilament protein antibody in the iPS group (A) and in the control group (B). (C and D) Immunohistochemistry for Tuj-1 antibody in the iPS group (C) and in the control group (D). (E and F) Immunohistochemistry for S-100 antibody in the iPS group (E) and in the control group (F). (G and H) Immunohistochemistry for GFAP antibody in the iPS group (G) and in the control group (H). Scale bar 50 μm. (I and J) Quantitative analysis of the total number of regenerative axons (I) and positive areas for S-100 antibody (J) assessed by morphometry software, Winroof. Bars and lines represent mean ± SD. $^{*}p < 0.05$.

withdrawal in the iPS group was significantly shorter than that in the control group at 4, 8 and 12 weeks, respectively (Fig. 3B). These results indicate that the nerve conduits coated with iPS cell-derived neurospheres accelerate the functional recovery of sciatic nerve gaps in mice.

3.3. Histological evaluation and histomorphometry

The luminal structure of the nerve conduits was maintained without collapse at 12 weeks after the implantation (Fig. 2B). Histologically, the luminal structure of the nerve conduits was also maintained at 12 weeks, and there were regenerative axons in the nerve conduits of both the control and iPS group, which were detected as positive for the neurofilament protein antibody (Fig. 4A and B). However, axon regeneration was greater in the iPS group than in the control. In particular, the immature neurons, which were positive for Tuj-1 antibody, were more prevalent in the iPS group than in the control group (Fig. 4C and D). The positive areas for S-100 antibody and GFAP antibody were larger in the iPS group than in the control group (Fig. 4E–H). In particular, the early Schwann cells, which were positive for GFAP antibody, were more prevalent in the iPS group (Fig. 4G and H). Morphometric analysis showed that the positive areas for the neurofilament protein and S-100 antibodies were significantly greater in the iPS group than in the control group (Fig. 4I and J). These results indicated that the nerve conduits coated with iPS cell-derived neurospheres accelerate the regeneration of peripheral nerves histologically in repairing sciatic nerve gaps in mice.

4. Discussion

There have been a few studies reporting the application of stem cells such as adipose-derived stem cells, hair follicle stem cells, bone marrow mesenchymal stem cells and ES cells to the regeneration of peripheral nerves using tissue engineered nerve conduits [20–26]. However, few reports have used a combination of iPS cells with nerve conduits to promote the re-growth of peripheral nerves. Wang et al. described tissue engineered nerve conduits fabricated by seeding neural crest stem cells (NCSC) derived from mixed-cultured human iPS cells and ES cells for sciatic nerve repair in rats [27]. The human ES cell is one of the typical pluripotent stem cells; however, since they are derived from a fertilized ovum, there are ethical issues that have yet to be resolved [28]. In addition, as the NCSC derived from human iPS cells and human ES cells were implanted into rats, there is still the problem of a heterograft. In contrast, in the present study, we used neurospheres that were derived solely from mouse iPS cells, and transplanted them into mice. Thus, relative to some prior experiments with interpretive limitations, the present study may be considered more clinically applicable. This is the first report to use iPS cells to reconstruct a defected peripheral nerve with nerve conduits.

In the present study, we used the secondary neurosphere derived from iPS cells for seeding of the nerve conduits. It has been reported that primary neurospheres mainly exhibit neurogenic differentiation potential, whereas passaged secondary neurospheres exhibit mainly gliogenic differentiation potential [8,14,29]. Since it has been known that Schwann cells play an important role in peripheral nerve regeneration with nerve conduits, the gliogenic secondary neurospheres, which have the ability to differentiate into Schwann cells, were selected [30,31]. Because the histologically positive areas for GFAP and S-100 antibodies were obviously increased in the iPS group at 12 weeks after implantation, we thought it was reasonable that the grafted iPS cell-derived neurospheres tended to differentiate into Schwann cells. In the iPS group, the positive areas for GFAP antibody were greater than those for S-100, indicating that the grafted iPS cell-derived neurospheres tended to differentiate into more immature and less mature myelinating Schwann cells, which might be responsible for formation of the myelin sheath or releasing nerve growth factor to promote regenerative axon growth. However, it remains unclear how the iPS cell-derived neurospheres tracked after implantation and how they promoted peripheral nerve regeneration. Further studies are necessary to elucidate the exact mechanisms, with respect to how the iPS-derived neurospheres promote the regeneration of peripheral nerves. On the other hand, the mature functional neurons, which had a large number of dendrite extensions, were not detected in the nerve conduits 12 weeks after implantation. The majority of cells on the conduit differentiated into immature neurons before developing into mature functional neurons, and these cells might play a role in axon extension.

One of the problems with using iPS cells for regenerative medicine is the potential for teratoma formation [13,32]. In the present study, we used the iPS-MEF-Ng-178B-5, which is a mouse iPS cell line that was established with three transcription factors, Oct3/4, Sox2 and Klf4 without a c-Myc [13]. Although these iPS cell-derived secondary neurospheres have tumorigenic potential, their tumorigenic propensity was very low, similar to that of secondary neurospheres from ES cells as previously described [9]. In that study, the tumor-forming propensity of secondary neurospheres generated from 36 mouse iPS cell lines derived in 11 different ways was evaluated; iPS-MEF-Ng-178B-5 were the least likely to cause tumorigenic transformation. Because tumor formation by iPS cell derivatives is affected by the iPS cells' tissue of origin and the methods used for reprogramming and differentiation, we used the same iPS-MEF-Ng-178B-5 and neural induction methods. At 12 weeks after implantation, no teratoma formation was found in the regenerated peripheral nerve. Longer follow-up studies of the present peripheral nerve repairing model using iPS cells are necessary for the evaluation of teratoma formation.

The potential benefits of the use of iPS cells for regenerative medicine, including personalized treatment, are immeasurable. The combination of iPS cell technology and bioabsorbable nerve conduits could represent a future tool for the treatment of peripheral nerve defects as an alternative to nerve autografts that avoids sacrificing intact nerves.

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