Long-term survival of transplanted induced pluripotent stem cell-derived neurospheres with nerve conduit into sciatic nerve defects in immunosuppressed mice

Takuya Yokoi, Takuya Uemura, Kiyohito Takamatsu, Ema Onode, Kosuke Shintani, Shunpei Hama, Yusuke Miyashima, Mitsuhiro Okada, Hiroaki Nakamura

Department of Orthopaedic Surgery, Osaka City University Graduate School of Medicine, Osaka, Japan

Department of Orthopaedic Surgery, Osaka General Hospital of West Japan Railway Company, Osaka, Japan

Department of Orthopaedic Surgery, Yodogawa Christian Hospital, Osaka, Japan

Department of Pediatric Orthopaedic Surgery, Osaka City General Hospital, Osaka, Japan

ARTICLE INFO

Keywords:
Induced pluripotent stem cells
Artificial nerve
Peripheral nerve
Regenerative medicine
Scaffold

ABSTRACT

Since the advent of induced pluripotent stem cells (iPSCs), clinical trials using iPSC-based cell transplantation therapy have been performed in various fields of regenerative medicine. We previously demonstrated that the transplantation of mouse iPSC-derived neurospheres containing neural stem/progenitor cells with bioabsorbable nerve conduits promoted nerve regeneration in the long term in murine sciatic nerve defect models. However, it remains unclear how long the grafted iPSC-derived neurospheres survived and worked after implantation. In this study, the long-term survival of the transplanted mouse iPSC-derived neurospheres with nerve conduits was evaluated in high-immunosuppressed or non-immunosuppressed mice using in vivo imaging for the development of iPSC-based cell therapy for peripheral nerve injury. Complete 5-mm long defects were created in the sciatic nerves of immunosuppressed and non-immunosuppressed mice and reconstructed using nerve conduits coated with iPSC-derived neurospheres labeled with ffLuc. The survival of mouse iPSC-derived neurospheres on nerve conduits was monitored using in vivo imaging. The transplanted iPSC-derived neurospheres with nerve conduits survived for 365 days after transplantation in the immunosuppressed allograft models, but only survived for at least 14 days in non-immunosuppressed allograft models. This is the first study to find the longest survival rate of stem cells with nerve conduits transplanted into the peripheral nerve defects using in vivo imaging and demonstrates the differences in graft survival rate between the immunosuppressed allograft model and immune responsive allograft model. In the future, if iPSC-derived neurospheres are successfully transplanted into peripheral nerve defects with nerve conduits using iPSC stock cells without eliciting an immune response, axonal regeneration will be induced due to the longstanding supportive effect of grafted cells on direct remyelination and/or secretion of trophic factors.

1. Introduction

Since the advent of induced pluripotent stem cells (iPSCs) in 2006, enormous progress has been made in stem cell biology and regenerative medicine [1,2]. Clinical trials using iPSC-based cell transplantation therapy are currently underway in various fields of regenerative medicine [3–5]. In previous studies, we investigated iPSC-based therapeutic approaches for peripheral nerve regeneration and demonstrated the efficacy and safety of the transplantation of iPSCs with tissue-engineered bioabsorbable nerve conduits [6–10]. In particular, we found that the transplantation of mouse iPSC-derived neurospheres containing neural stem/progenitor cells, with nerve conduits promoted nerve regeneration in the long term in murine sciatic nerve defect models [8]. However, it remains unclear how long the grafted iPSC-derived neurospheres survived and worked after implantation. The aim of this study was to evaluate the survival of transplanted mouse iPSC-derived neurospheres with nerve conduits in sciatic nerve defects of highly immunosuppressed or non-immunosuppressed mice using in vivo imaging. This study builds...
on preclinical research on the development of iPSC-based cell therapy for the treatment of peripheral nerve injury.

2. Materials and methods

2.1. Neural induction and lentivirus transduction of iPSCs

Mouse iPSCs from the iPS-MEF-Ng-178B-5 cell line were provided by RIKEN BRC via the National Bio-Resource Project of MEXT, Japan [11]. The culturing and neural induction of iPSCs were performed as described previously [6–10,12]. Briefly, secondary neurospheres containing neural stem/progenitor cells were derived from iPSCs via embryonic body formation and were infected with a lentivirus expressing ffLuc for bioluminescence imaging of the graft [13–16]. ffluc a green fluorescence protein (modified from Venus) fused to a luminescence protein (Luciferase 2), under the control of an elongation factor (EF) promoter (pCSII-EF-Venus-Luc2).

2.2. Preparation of nerve conduits coated with iPSC-derived neurospheres

The nerve conduit (outer diameter: 2 mm; inner diameter: 1 mm; length: 7 mm) used in this study was identical to those used for the treatment of sciatic nerve defects in our previous studies, which showed consistent axonal regeneration [7–10,17] (Fig. 1). This bioabsorbable nerve conduit consists of two layers: an outer layer of a poly(ε-caprolactone) (PCL) (50:50) sponge copolymer. The PLA and PCL copolymer sponge of the inner layer has a honeycomb structure with pore sizes of 10–50 μm, into which regeneration-facilitating cells, such as iPSC-derived neurospheres, can enter and proliferate as a scaffold [6–8].

Day 7 iPSC-derived secondary neurospheres labeled with the ffluc lentivirus were dissociated into single cells and were carefully seeded over each nerve conduit at a density of 2.0 × 10⁶ cells per conduit, as previously described [7–10]. These nerve conduits were placed in Dulbecco’s modified Eagle’s medium supplemented with 10% embryonic stem cell-qualified fetal bovine serum (all from Gibco Life Technologies, California, USA) for 14 days. This ensured that the nerve conduits were three-dimensionally coated with iPSC-derived secondary neurospheres, which were histologically differentiated into Schwann-like cells, playing the most important role in peripheral nerve regeneration, as previously described [6,8].

2.3. Transplantation of nerve conduits coated with iPSC-derived neurospheres in murine sciatic nerve defects

The experiments were carried out in strict accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals of Osaka City University. Male C57BL/6 mice (6 weeks old, n = 3), used as non-immunosuppressed mice, and NOD/SCID mice (6 weeks old, n = 3), used as highly immunosuppressed mice, were purchased from Japan SLC (Hamamatsu, Japan) and housed in an air-conditioned room with free access to food and water. Complete 5-mm long defects were created in the left sciatic nerves of each mouse and reconstructed using nerve conduits coated with iPSC-derived neurospheres labeled with the ffluc (7–17) (Fig. 1). Both the proximal and distal stumps of the sciatic nerve were pulled 1 mm into the nerve conduit. The nerve ends were sutured to the lumen wall of the inner layer under a microscope.

2.4. Bioluminescence imaging analysis

After transplantation, the survival of mouse iPSC-derived neurospheres on the nerve conduits was continuously monitored in both C57BL/6 and NOD/SCID mice using in vivo imaging. Mice were anesthetized using isoflurane and the hair at the surgical implantation site was shaved. After exploring the nerve conduit, a luciferase substrate, d-luciferin (PerkinElmer, Waltham, Massachusetts, USA), was directly infused (50 μL) into the nerve conduit, and the ffluc-labeled cells were monitored using an In Vivo Imaging System (IVIS) Spectrum instrument and a CCD optical macroscopic imaging system (PerkinElmer, Waltham, Massachusetts, USA). The bioluminescence signals were observed using the luminescent imaging mode (exposure time: 1 min, field of view: 5 cm²) and measured 0, 4, 7, 14, 28, 56, 84, 168, and 365 days after transplantation.

3. Results

Although all mice survived for 168 days after transplantation, two NOD/SCID mice and a C57BL/6 mouse died 365 days after transplantation. The images of all mice with and without bioluminescence signals and quantitative analyses of the photon counts over time, using cell tracing in IVIS, are shown in Fig. 2. A temporary increase in the iPSC-derived neurospheres with nerve conduits transplanted into sciatic nerve defects was observed, which peaked 7 days after transplantation.

Fig. 1. A: Gross appearance of the bioabsorbable tubular nerve conduit. B: The nerve conduit coated with the iPSC-derived neurospheres labeled with and without the ffluc lentivirus before implantation. C: The 5-mm defects of sciatic nerve were reconstructed with the nerve conduit coated with iPSC-derived neurospheres.
in both C57BL/6 and NOD/SCID mice (Fig. 2C). This was followed by a gradual decrease in neurospheres in both mice. However, while the neurospheres in the C57BL/6 mice survived for at least 14 days, and eventually disappeared 28 days after transplantation, the neurospheres in the NOD/SCID mice survived for as long as 365 days after transplantation.

4. Discussion

In this study, iPSC-derived neurospheres with nerve conduits were
found to survive for up to one year after transplantation in immunosuppressed mice. This study is the first to confirm the longest survival rate of transplanted stem cells with nerve conduits using in vivo imaging and demonstrates the differences in graft survival rate between immunosuppressed allograft models and immune responsive allograft models, based on previous in vivo studies on stem cell-based cell transplantation with nerve conduits for peripheral nerve regeneration [16,18,19]. This result is key for the initiation of the first in-human clinical application of iPSC-based cell therapy for peripheral nerve injury. Similar to an ongoing clinical trial for spinal cord injury, we believe that the allograft model using human iPSCs matching the patient’s HLA type from the iPSC stocks is more reasonable than the autograft model using the iPSC derived from the patient’s own cells because autologous iPSC transplants are costly and take a long time to establish and differentiate [5, 20–23].

Previously, in vivo studies on iPSC-based cell transplantation therapy have been conducted for the treatment of peripheral nerve defects using iPSC-derived neural stem cells with nerve conduits [16,24–27]. With regard to the graft survival rate, transplanted iPSC-derived neural crest stem cells or neural stem/progenitor cells with nerve conduits in a xenograft model of athymic nude rats were reported to survive histologically for up to 4 to 8 weeks after transplantation [24,32]. In a xenograft model of highly immunosuppressed NOD/SCID mice, stem cells purified from human iPSC-derived neural crest-like cells survived up to 12 weeks after transplantation, as confirmed by in vivo imaging [16]. However, no long-term follow-up studies have been conducted, such that the percentage and duration of surviving iPSC-derived neural crest stem cells with nerve conduits after transplantation into sciatic nerve defects remains to be fully elucidated.

We have consistently used iPSC-derived neural spheres containing neural stem/progenitor cells because these cells are the most effectively established and most strictly evaluated cells in translational preclinical studies for the development of iPSC-based cell therapy for the treatment of spinal cord injuries [5,12,13,20,28,29]. With regard to their graft survival rate, in an allograft model of spinal cord injury in non-immunosuppressed C57BL/6 mice, the graft survival rate of the same mouse iPSC-derived neural spheres as those used in this study was 18% at 5 weeks after transplantation, as confirmed by in vivo imaging [13]. In a xenograft model of spinal cord injury in immunosuppressed NOD/SCID mice, human iPSC-derived neural spheres were found to survive immunohistochemically for up to 16 weeks after transplantation [28]. In the present study, the longest graft survival was confirmed by in vivo imaging. The graft survival rate of mouse iPSC-derived neural spheres was approximately 16% at 1 year after transplantation in an allograft model for peripheral nerve defects in immunosuppressed NOD/SCID mice. The differences in the long-term survival rate of transplanted mouse and human iPSC-derived neural spheres depend on transplantation models - allograft or xenograft models. The survival rate in the allograft model was higher than that in the xenograft model, even if the mouse iPSC-derived neural spheres were transplanted in the immunosuppressed NOD/SCID mice, because the immune response remained at an exceedingly small degree in NOD/SCID mice with natural killer cells, macrophages, and complements exerting partial activity [30]. Thus, in a xenograft model using the immunosuppressed NOD/SCID mice, human iPSC-derived neural spheres survived for up to 16 weeks after transplantation [28]. On the other hand, in this study of an allograft model using the same NOD/SCID mice, mouse iPSC-derived neural spheres survived for up to 365 days after transplantation. In this study, there might be the possibility of contamination of undifferentiated iPSCs in the neural spheres. However, it was highly unlikely because we used more and more differentiated iPSCs. Firstly, the secondary neural spheres that were passaged from the primary neural spheres derived from undifferentiated iPSCs were suspended in the nerve conduit. Secondly, the secondary neural spheres were differentiated in the nerve conduit for more than two weeks and eventually differentiated into cells that contained GFAP-positive immature glial cells, S-100-positive mature Schwann-like cells and Tuj 1-positive primitive neurons. Thirdly, there was no obvious teratoma formation, which is usually formed from the contamination of undifferentiated iPSCs in the long-term, sciatic nerve defect model [8].

In this study, the transplanted iPSC-derived neural spheres in C57BL/6 mice completely disappeared as early as 4 weeks after transplantation due to allograft rejection because the grafted mouse iPSCs (iPS-MEF-Ng-1788S-5) did not originate from the same C57BL/6 strain as the recipient mice. Briefly, the iPS-MEF-Ng-1788S-5 cell line was generated from fibroblasts derived from F1 hybrid mice of the 129S6 and C57BL/6 strains [11,31]. On the other hand, iPSC-derived neural spheres transplanted into NOD/SCID mice survived as long as 1 year due to high immunosuppression. However, even in the highly immunosuppressed allograft model, the transplanted cells decreased gradually over time. This was due to the fact that the immune response remained negligible in NOD/SCID mice, with natural killer cells, macrophages, and complements exerting partial activity [30]. In the future, iPSC-derived neural spheres with nerve conduits transplanted successfully into peripheral nerve defects in more complete immunosuppressed model, such as using iPSC stock cells, could be used in xenograft models over a longer time compared with the longstanding supportive effect of the grafted cells, including direct cell replacement effect on remyelination and indirect trophic effect.

Acknowledgment

The authors thank Masaya Nakamura MD, PhD, Kazuki Sato MD, PhD, Narihito Nagoshi MD, PhD, (Department of Orthopaedic Surgery, Keio University School of Medicine, Japan), Hideyuki Okano MD, PhD, and ShinSUKE Shibata MD, PhD (Department of Physiology, Keio University School of Medicine, Japan) for material transfer of the EF promoter (pCSII-EF-IVenus-Luc2) for the lentivirus expressing fLuc, in addition to their excellent assistance with neural induction of iPSCs. This work was supported by Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Number JP16K10872. We would like to thank Editage (www.editage.com) for English language editing.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.jbrep.2021.100979

References

[1] Y. Shi, H. Inoue, J.C. Wu, S. Yamanaka, Induced pluripotent stem cell technology: a decade of progress, Nat. Rev. Drug Discov. 16 (2017) 115–130, https://doi.org/10.1038/nrd.2016.245.
[2] K. Takahashi, S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, Cell 126 (2006) 663–676, https://doi.org/10.1016/j.jclineed.2006.07.024. S0092-8674(06)00976-7 [pii].
[3] M. Mandai, A. Watanabe, Y. Kurimoto, Y. Hirami, C. Morinaga, T. Daimon, M. Fujihara, H. Akimaru, N. Sakai, Y. Shibata, M. Terada, Y. Nomiya, S. Tanishima, M. Nakamura, H. Kamaso, S. Sugita, A. Onishi, T. Ito, R. Fujita, S. Kawamata, M. J. Go, C. Shinohara, K.I. Hata, M. Sawada, M. Yamamoto, S. Ohta, Y. Ohara, K. Yoshida, J. Kuwahara, Y. Kitano, N. Amano, M. Umekage, F. Kitaoka, A. Tanaka, C. Okada, N. Takasu, S. Ogawa, S. Yamanaka, M. Takahashi, Autologous induced stem-cell-derived retinal cells for macular degeneration, N. Engl. J. Med. 376 (2017) 1038–1046, https://doi.org/10.1056/NEJMoa1608368.
[4] S. Miyagawa, Y. Sawa, Building a new strategy for treating heart failure using induced pluripotent stem cells, J. Cardioi. 72 (2018) 445–448, https://doi.org/10.1016/j.jcc.2018.05.002.
[5] O. Tsuji, K. Sugai, R. Yamaguchi, S. Tashiro, N. Nagoshi, J. Koyama, T. Iida, T. Ohkubo, G. Ikekura, M. Ido, M. Shinohara, K. Fujiyoshi, Y. Kanemura, S. Yamanaka, M. Nakamura, H. Okano, Concise review: laying the groundwork for a first-in-human study of an induced pluripotent stem cell-based intervention for spinal cord injury, Stem Cell. 37 (2019) 6–13, https://doi.org/10.1002/stem.2926.
[6] T. Uemura, K. Takamatsu, M. Ikeda, M. Okada, K. Kazuki, Y. Ikada, H. Nakamura, A tissue-engineered bioabsorbable nerve conduit created by three-dimensional culture of induced pluripotent stem cell-derived neurons, Bio Med. Mater. Eng. 21 (2011) 333–339, https://doi.org/10.3233/BME-2012-0680.
[7] T. Uemura, K. Takamatsu, M. Ikeda, M. Okada, K. Kazuki, Y. Ikada, H. Nakamura, Transplantation of induced pluripotent stem cell-derived neurons for
peripheral nerve repair, Biochem. Biophys. Res. Commun. 419 (2012) 130–135, https://doi.org/10.1016/j.bbrc.2012.01.154.

[8] T. Uemura, M. Ikeda, K. Takamatsu, T. Yokoi, M. Okada, H. Nakamura, Long-term efficacy and safety outcomes of transplantation of induced pluripotent stem cell-derived neurospheres with bioabsorbable nerve conduits for peripheral nerve regeneration in mice, Cells Tissues Organs 200 (2014) 78–91, https://doi.org/10.1159/000370322.

[9] M. Ikeda, T. Uemura, K. Takamatsu, M. Okada, K. Kazuki, Y. Tabata, Y. Ikada, H. Nakamura, Acceleration of peripheral nerve regeneration using nerve conduits in combination with induced pluripotent stem cell technology and a basic fibroblast growth factor drug delivery system, J. Biomed. Mater. Res. 102 (2014) 1370–1378, https://doi.org/10.1002/jbm.b.34816.

[10] Y. Yokoi, T. Uemura, K. Takamatsu, K. Shintani, E. Onode, M. Okada, N. Hitakana, H. Nakamura, Bioabsorbable nerve conduits coated with induced pluripotent stem cell-derived neurospheres enhance axonal regeneration in sciatic nerve defects in aged mice, J. Biomed. Mater. Res. B Appl. Biomater. 106 (2018) 1752–1758, https://doi.org/10.1002/jbmr.33983.

[11] M. Nakagawa, M. Koyanagi, K. Tanabe, K. Takahashi, T. Ishikawa, T. Aoi, K. Okita, Y. Mochiduki, N. Takizawa, S. Yamanaka, Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts, Nat. Biotechnol. 26 (2008) 101–106, https://doi.org/10.1038/nbt1174.

[12] K. Miura, Y. Okada, T. Aoi, A. Okada, K. Takahashi, K. Okita, M. Nakagawa, M. Koyanagi, K. Tanabe, M. Ohnuki, D. Ogawa, E. Ikeda, H. Okano, S. Yamanaka, Variation in the safety of induced pluripotent stem cell lines, Nat. Biotechnol. 27 (2009) 743–745, https://doi.org/10.1038/nbt.1554.

[13] O. Tsuji, K. Miura, Y. Okada, K. Fujiyoshi, M. Mukaino, N. Nagoshi, K. Kitamura, G. Kumagai, M. Nishino, S. Tomisato, H. Higashi, T. Naga, H. Katoh, K. Kobuda, Y. Matsuuchi, M. Yuzaki, E. Ikeda, Y. Toyama, M. Nakamura, S. Yamanaka, H. Okano, Therapeutic potential of appropriately evaluated safe-induced pluripotent stem cells for spinal cord injury, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 12704–12709, https://doi.org/10.1073/pnas.0910106107.

[14] C. Harayama, T. Tsuji, A. Hanay, S. Okada, A. Yasuda, T. Fukano, A. Akazawa, M. Nakamura, K. Umamaheswara, M. Yuzaki, E. Toyama, M. Nakamura, S. Yamanaka, G. Itakura, H. Iwai, S. Nishimura, A. Yasuda, S. Okano, Essential fluorescent protein expression in mouse ES cells, embryos, and adult animals, Genesis 37 (2005) 191, https://doi.org/10.1002/term.2823.

[15] N. Hikishima, T. Konomi, K. Fujiyoshi, O. Tsuji, Y. Toyama, S. Yamanaka, M. Nakamura, R. Tsuchiya, Uncultured adipose-derived regenerative cells promote peripheral nerve regeneration, Regen. Ther. 11 (2019) 75–80, https://doi.org/10.1016/j.regther.2019.05.006.

[16] M. Umekage, Y. Sato, N. Takasu, Overview: an iPS cell stock at CiRA, Inflamm. Cell 23 (2019) 1758, https://doi.org/10.1016/j.jcm.2019.05.006.

[17] N. Nagoshi, O. Tsuji, M. Nakamura, H. Okano, Cell therapy for spinal cord injury using induced pluripotent stem cells, Regen. Ther. 11 (2019) 75–80, https://doi.org/10.1016/j.regther.2019.05.006.

[18] M. Nakamura, H. Okano, Cell transplantation therapies for spinal cord injury focusing on induced pluripotent stem cells, Cell Res. 23 (2013) 70–80, https://doi.org/10.1038/cr.2013.171.

[19] H. Okano, S. Yamanaka, iPS cell technologies: significance and applications to CNS regeneration and disease, Mol. Brain 7 (2014) 22, https://doi.org/10.1186/s12215-014-0159-9.

[20] H. Tsuchiya, Uncultured adipose-derived regenerative cells promote peripheral nerve regeneration in rats, Sci Rep. 11 (1) (2021) 4204, https://doi.org/10.1038/s41598-021-83385-9.