



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

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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

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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 embryo 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(L-lactide) (PLA) multifilament fiber mesh and an inner layer of a PLA/polycaprolactone (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 2.0×10^6 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* [7–10,17] (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 cm^2) 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,

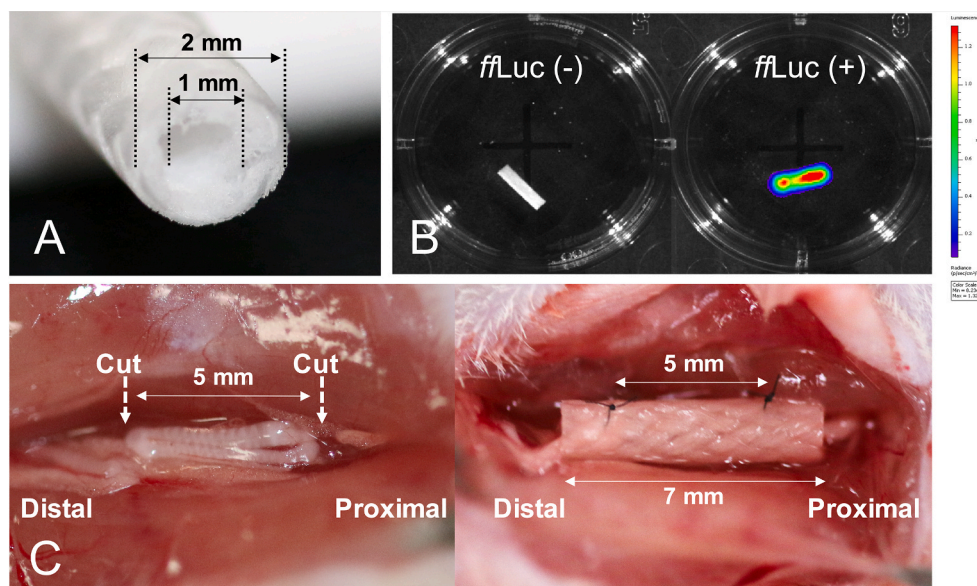


Fig. 1. A: Gross appearance of the bioabsorbable tubular nerve conduit. B: The nerve conduit coated with the iPSC-derived neurospheres labeled with and without the *ffLuc* lentivirus before implantation. C: The 5-mm defects of sciatic nerve were reconstructed with the nerve conduit coated with iPSC-derived neurospheres.

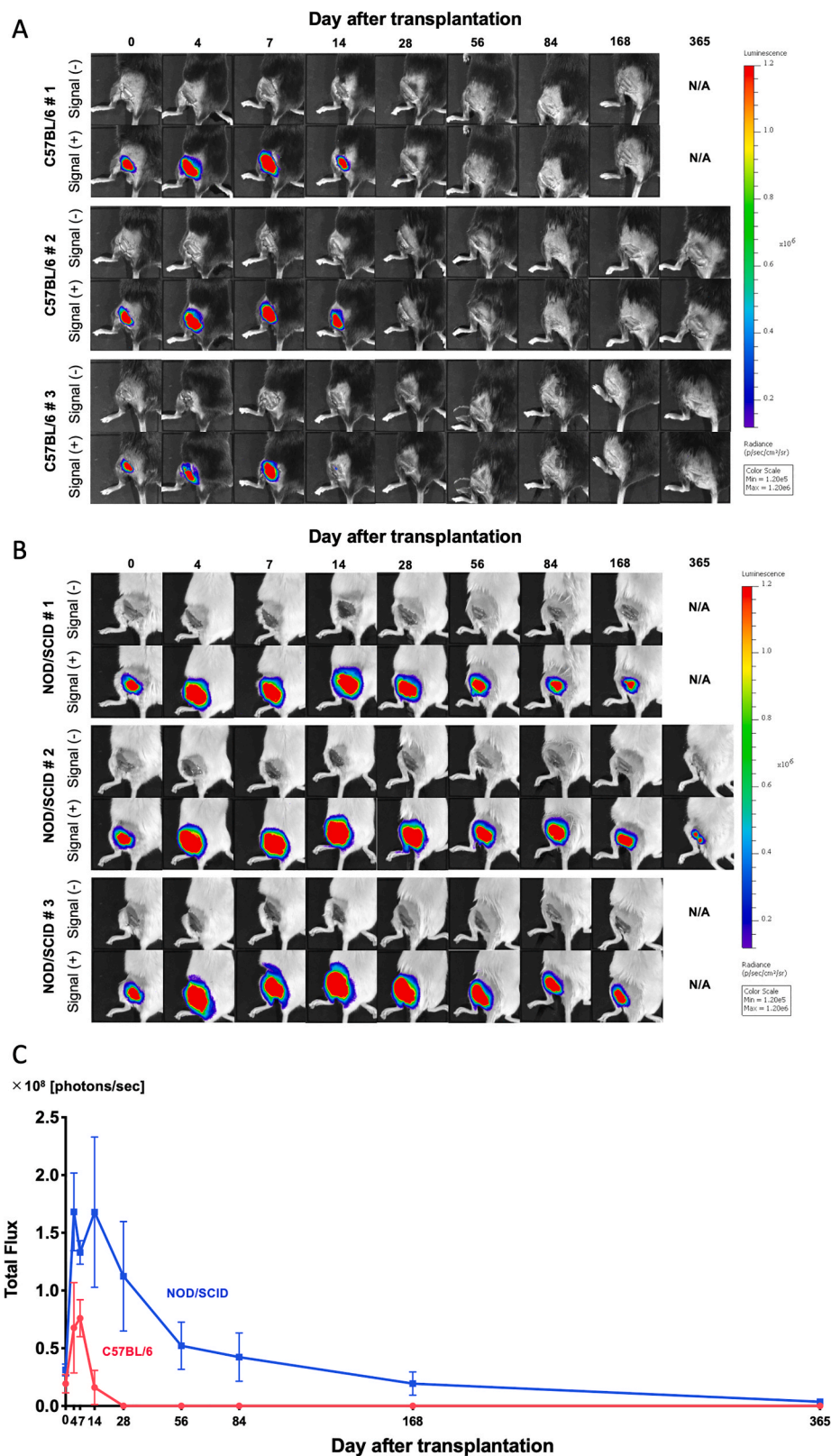


Fig. 2. Images (A and B) and quantitative analyses of photon counts (C) in the *in vivo* imaging. Bars and lines represent the mean ± standard deviation (SD).

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 crest 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 neurospheres 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 neurospheres 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 neurospheres 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 neurospheres 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 neurospheres 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 neurospheres 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 neurospheres 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 neurospheres survived longer (for 365 days after transplantation). In this study, there might be the possibility of contamination of undifferentiated iPSCs in the neurospheres. However, it was highly unlikely because we used more and more differentiated iPSCs. Firstly, the secondary neurospheres that were passaged from the primary neurospheres derived from undifferentiated iPSCs were suspended in the nerve conduit. Secondly, the secondary neurospheres 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 neurospheres 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-178B-5) did not originate from the same C57BL/6 strain as the recipient mice. Briefly, the iPS-MEF-Ng-178B-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 neurospheres 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 neurospheres with nerve conduits transplanted successfully into peripheral nerve defects in a more complete immunosuppressed model, such as using iPSC stock cells, could result in axonal regeneration owing to the longstanding supportive effect of the grafted cells, including direct cell replacement effect on remyelination and indirect trophic effect.

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