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Induced pluripotent stem cells for neural tissue engineering

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ABSTRACT

Induced pluripotent stem cells (iPSCs) hold great promise for cell therapies and tissue engineering. Neural crest stem cells (NCSCs) are multipotent and represent a valuable system to investigate iPSC differentiation and therapeutic potential. Here we derived NCSCs from human iPSCs and embryonic stem cells (ESCs), and investigated the potential of NCSCs for neural tissue engineering. The differentiation of iPSCs and the expansion of derived NCSCs varied in different cell lines, but all NCSC lines were capable of differentiating into mesodermal and ectodermal lineages, including neural cells. Tissue-engineered nerve conduits were fabricated by seeding NCSCs into nanofibrous tubular scaffolds, and used as a bridge for transected sciatic nerves in a rat model. Electrophysiological analysis showed that only NCSC-engrafted nerve conduits resulted in an accelerated regeneration of sciatic nerves at 1 month. Histological analysis demonstrated that NCSC transplantation promoted axonal myelination. Furthermore, NCSCs differentiated into Schwann cells and were integrated into the myelin sheath around axons. No teratoma formation was observed for up to 1 year after NCSC transplantation *in vivo*. This study demonstrates that iPSC-derived multipotent NCSCs can be directly used for tissue engineering and that the approach that combines stem cells and scaffolds has tremendous potential for regenerative medicine applications.

1. Introduction

Cell source is a major issue for tissue engineering and regenerative medicine. An exciting breakthrough in stem cell biology is that adult somatic cells (e.g., skin fibroblasts) can be reprogrammed into induced pluripotent stem cells (iPSCs) by the activation of a limited number of genes (transgenes) such as Oct3/4, Sox2, c-Myc and KLF4 [1,2] or Oct3/4, Sox2, Nanog and Lin28 [3]. The iPSCs derived from somatic cells make it possible for patient-specific cell therapies, which bypasses immune rejection issue and ethical concerns of deriving and using embryonic stem cells (ESCs) as a cell source. The unlimited expansion potential of iPSCs also makes them a valuable cell source for tissue engineering. However, to use iPSCs as a cell source, many important issues remain to be addressed, such as the differences among various iPSC lines in differentiation and expansion and the appropriate differentiation stage of the cells for specific tissue engineering applications. Neural crest stem cells (NCSCs) can differentiate into cell types of all three germ layers, and represent a valuable model system to investigate the differentiation and therapeutic potential of stem cells [4–8]. Here we derived NCSCs from human iPSCs and ESCs to determine the variation among iPSC lines, and used the model of peripheral nerve regeneration to investigate the differentiation and therapeutic potential of NCSCs *in vivo*.

Nerve conduits are usually used to bridge transected peripheral nerves [9,10]; however, the regeneration is often limited and slow [11–14]. There is evidence that the transplantation of Schwann cells or precursor cells derived from skin or other adult tissues can facilitate nerve regeneration [15,16], but adult cell sources are limited by the number of cells that can be obtained and complicated by the need to sacrifice additional nerves and tissues. Moreover, there is a lack of efficiency and consistency in cell isolation and expansion, which causes variability in therapeutic

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efficacy. In contrast, NCSCs derived from iPSCs can be immune compatible, expandable, and well characterized as a valuable cell source for the regeneration of peripheral nerve and other tissues.

2. Materials and methods

(Details can be found in Supplemental Methods)

2.1. Cell culture, NCSC derivation and differentiation

Undifferentiated human iPSCs (passages 22–45) and hESC lines H1 and H9 (WiCell Research Institute; passages 30–50) were maintained as described previously [17]. To derive NCSCs, iPSCs and ESCs were detached by collganase IV (1 mg/ml) and dispase (0.5 mg/ml), and grown as embryo body (EB)-like floating cell aggregates in ESC maintenance medium without bFGF for 5 days. The cell aggregates were allowed to adhere to CELLstart matrix (Invitrogen) coated dishes in a serum-free neural induction medium (SFM) consisting of Knockout DMEM/F12, StemPro neural supplement (Invitrogen), 1% GlutaMAX™-I (Invitrogen), 20 ng/ml bFGF and 20 ng/ml EGF. After 7 more days, the colonies with rosette structures were mechanically harvested, cultured in suspension in SFM medium for one week, replated onto CellStart-coated dishes, and cultured for 3 more days. Cells were dissociated into single cells by TryplE Select (Invitrogen) and cultured in SFM medium in ultra low attachment tissue culture plates (Costar) for one more week. The secondary spheres formed in suspension were collected and replated on CellStart-coated dishes in SFM medium. Cells were allowed to migrate out of the attached secondary spheres and grow to confluence; then the cells were dissociated and cultured as a monolayer. NCSC lines that were homogeneously positive for p75 were selected for further studies. Some of the cell lines were further purified by FACS for p75 + cells to obtain homogeneous populations that were positive for p75, HNK1 and AP2. The cells were maintained in SFM on CellStart-coated dishes with 50% of the medium changed every 2 days, and were passaged weekly. To test the multipotency of NCSCs, cell differentiation into neural cells and mesenchymal cells was carried out using the protocol described previously [7,18].

2.2. Biochemical analysis

Immunostaining, microscopy, real-time polymerase chain reaction (PCR) and flow cytometry analysis were performed as described previously [18,19].

2.3. Scaffold fabrication

Electrospinning technique was used to produce nanofibrous nerve conduits. Nonwoven aligned nanofibrous nerve conduits composed of poly(L-lactide-co-caprolactone) (70:30, Purac Biomaterials, Amsterdam, Netherlands), poly(propylene glycol) (Acros Organics, Morris Plains, NJ) and sodium acetate (Sigma, St. Louis, MO) were fabricated by using a customized electrospinning process. To make tubular scaffolds with aligned nanofibers in the longitudinal direction on luminal surface, a rotating mandrel assembly with two electrically conductive ends and a central non-conductive section was used. The jet stream of polymer solution from the spinneret whipped between the two conductive ends, resulting in longitudinally aligned nanofibers forming a tubular scaffold on the non-conductive portion of the mandrel. To enhance the mechanical strength of the scaffolds, outer layers of random nanofibers were deposited on this layer of longitudinally aligned fibers.

2.4. Tissue engineered conduits and in vivo transplantation

The nerve conduits were sterilized by ethylene oxide gas sterilization before use. NCSCs were detached and re-suspended in SFM ($2–3 \times 10^4$ cells/μl). The cell suspension was mixed with a cold matrigel solution at a 2:1 ratio (volume to volume), and injected into the nanofibrous nerve conduits (e.g., 45 μl for one conduit 1.2 cm in length). The tissue-engineered constructs were kept in the incubator for 1 h, and NCSC maintenance medium was added to cover the constructs. The culture was maintained in the incubator overnight before surgery. Live/Dead assay (Molecular Probes) was used to assess the viability of the NCSCs in the tissue-engineered nerve conduits.

All experimental procedures with animals were approved by the ACUC committee at UC Berkeley and were carried out according to the institutional guidelines. Adult female athymic rats (National Cancer Institute) weighing 200–250 g were used in all experiments. Three experimental groups were included: (1) conduits filled with matrigel (diluted with SFM at a 1:2 ratio) without cells (as control, 10 animals), (2) conduits seeded with NCSCs derived from iPSCs (4 iPSC lines: BJ1-iPS1, ADAfE4-iPS38, dH1f-iPS2-2, MSC-iPS1; 6 animals for each cell line), and (3) conduits seeded with NCSCs derived from hESCs (H1 and H9 cell lines).

The sciatic nerve was severed with a scalpel, and 0.6 cm of the nerve trunk was cut off. A nerve conduit (1.2 cm in length) was inserted between the two nerve stumps and sutured under the microscope with 9-0 sutures, creating a gap of 1 cm between the two stumps.

2.5. Electrophysiology

To directly evaluate the regeneration of the sciatic nerve across the conduits, electrophysiology testing was performed before euthanasia. Electrical stimuli (1 mA in strength) were applied to the native sciatic nerve trunk at the point 5 mm proximal to the graft suturing point, and CMAPs were recorded on the gastrocnemius belly. Normal CMAPs from the un-operated contralateral side of sciatic nerve were also recorded for comparison.

2.6. Histological analysis

After electrophysiology testing, the nerve conduits were explanted and fixed in 4% paraformaldehyde at 4 °C. Longitudinal sections (12 μm in thickness) and cross sections in the middle portion of the graft (10 μm in thickness; 5–7 mm from the proximal end of the graft) were cryosectioned for H&E staining and immunostaining.

2.7. Evaluation of myelination

The nerve conduits were explanted, and nerve cross sections at the middle portion of the graft (5–7 mm from the proximal end) were processed and stained by toluidine blue for myelin sheath. Briefly, the samples were fixed, mounted in embedding resin, sectioned at 800 nm thickness with a microtome, and stained with 1% toluidine blue. The total number of myelinated axons per unit area was quantified by using ImageJ software.

3. Results

3.1. Derivation and characterization of NCSCs from iPSCs and hESCs

We derived NCSCs from ESCs or iPSCs as described in Fig. 1A. To derive NCSCs, hESCs and iPSCs were first cultured as cell aggregates in suspension, and then allowed to adhere to form rosette structures (Fig. 1B) for NCSC isolation. Two human ESC lines (H1 and H9) robustly differentiated into NCSCs. Out of seven human iPSC lines tested, MRC5-iPS7 (derived from MRC5 human fetal lung fibroblasts) and hFib2-iPS4 cells (derived from skin fibroblasts) [2] did not differentiate effectively and maintained an undifferentiated ESC/iPSC morphology (Fig. 1C,D), while the other five iPSC lines gave rise to NCSCs. For these five iPSC lines, three were derived from skin fibroblasts (BJ1-iPS1, ADAfE4-iPS38, ADAfE4-iPS38-2), one was derived from H1-OGN differentiated fibroblasts (dH1f-iPS2-2), and one was derived from bone marrow mesenchymal stem cells (MSC-iPS1) [2]. These results suggest that the differentiation of different iPSC lines might vary.

At the rosette stage of ESC and iPSC differentiation, four types of colonies were observed, i.e., colonies with rosette structure (Fig. 1B), colonies with differentiated neurons (Fig. 1E,H), colonies with myofibroblasts that were positive for smooth muscle α-actin (Fig. 1F,I), and a small number of undifferentiated colonies that were positive for Oct3/4 (Fig. 1G,J). The percentage of each type of colonies for each ESC/iPSC line is summarized in Supplementary Fig. 1. Some iPSC lines (BJ1-iPS1, MSC-iPS1, ADAfE4-iPS38-2) and ESC line (H9) formed more colonies with the rosette structure, whereas other iPSC line (dH1f-iPS2-2, ADAfE4-iPS38) and ESC line (H1) had lower number of colonies with rosettes.

Although different iPSC and ESC lines had different efficiency in forming rosettes, the majority of cells in the colonies with rosette structures were positive for neural crest markers AP2, nestin and p75 (Fig. 1K–M). ESC and iPSC colonies with rosette structures were mechanically harvested, cultured in suspension, and transferred to monolayer culture. Flow cytometry analysis of the cells showed that 80–95% of cells were p75+/HNK1+ (Supplemental Fig. 2) in different NCSC lines. The gene expression at different stages of the differentiation process was characterized. ESCs (as exemplified in Fig. 2A) lost the expression of pluripotency markers Oct3/4 and Nanog upon differentiation. The expression of mesoderm marker T-Brachyury and endoderm marker FoxA2 also decreased. In contrast, the expression of various neural crest markers including