Biochimica et Biophysica Acta 1850 (2015) 22–32

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Microencapsulation of dopamine neurons derived from human induced pluripotent stem cells

Shuhei Konagaya, Hiroo Iwata *

Institute for Frontier Medical Sciences, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

ARTICLE INFO

Article history:
Received 22 April 2014
Received in revised form 10 September 2014
Accepted 24 September 2014
Available online 1 October 2014

Keywords:
Induced pluripotent stem cell
Dopamine neuron
Microencapsulation

ABSTRACT

Background: Dopamine neurons derived from induced pluripotent stem cells have been widely studied for the treatment of Parkinson's disease. However, various difficulties remain to be overcome, such as tumor formation, fragility of dopamine neurons, difficulty in handling large numbers of dopamine neurons, and immune reactions. In this study, human induced pluripotent stem cell-derived precursors of dopamine neurons were encapsulated in agarose microbeads. Dopamine neurons in microbeads could be handled without specific protocols, because the microbeads protected the fragile dopamine neurons from mechanical stress.
Methods: hiPS cells were seeded on a Matrigel-coated dish and cultured to induce differentiation into a dopamine neuronal linage. On day 18 of culture, cells were collected from the culture dishes and seeded into U-bottom 96-well plates to induce cell aggregate formation. After 5 days, cell aggregates were collected from the plates and microencapsulated in agarose microbeads. The microencapsulated aggregates were cultured for an additional 45 days to induce maturation of dopamine neurons.
Results: Approximately 60% of all cells differentiated into tyrosine hydroxylase-positive neurons in agarose microbeads. The cells released dopamine for more than 40 days. In addition, microbeads containing cells could be cryopreserved.
Conclusion: hiPS cells were successfully differentiated into dopamine neurons in agarose microbeads.
General significance: Agarose microencapsulation provides a good supporting environment for the preparation and storage of dopamine neurons.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disease mainly caused by selective loss of dopamine (DA) neurons in the substantia nigra [1]. L-3,4-Dihydroxyphenylalaninedopa and DA receptor agonists have been administered to patients in pharmaceutical treatments of PD [2–4]. Unfortunately, these treatments cannot always be implemented for long periods due to their side effects, such as dyskinesia, and the efficacy of these treatments gradually decreases. In recent years, cell replacement therapy has been considered as an alternative method to treat PD. Brain tissues from aborted fetuses have been transplanted into PD patients. Methods for engrafting DA neurons and the associated physiological recoveries have differed between reports [5–7], possibly due to immature DA neurons in the fetal brain tissue or to an insufficient number of DA neurons in the graft. In addition, the shortage of donors and ethical concerns make it difficult to accept cell replacement therapy as the normal course of treatment for PD.

Pluripotent stem cells, such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, have been regarded as new sources for cell transplantation therapy [8,9]. The cells can expand in number without limit under the undifferentiated condition, and can be differentiated into multiple cell types. Differentiation protocols to turn pluripotent cells into DA neurons have been reported [10–12]. Differentiated DA neurons from ES cells and iPS cells were previously transplanted into PD model animals to demonstrate the efficacy of these cells in the treatment of PD [10–12].

Although DA neurons derived from pluripotent stem cells are considered to have utility in human PD patients, various difficulties still remain to be overcome. First, tumor formation must be carefully considered in cell transplantation therapy using iPS-derived cells [13,14]. Undifferentiated pluripotent stem cells and neural progenitors contained in transplants may proliferate and overgrow in the host brain [15]. It has been claimed that the risk of tumor formation can be reduced by maturing cells for a long period in vitro before transplantation [16,17]. Second, graft rejection by the host immune system is unavoidable in transplantation therapy and should be carefully controlled. When DA neurons derived from ES cells are used, the graft is expected to be recognized as allogeneic tissue and thus will be rejected by the host immune system [18,19]. Even when iPS cells are derived from a

* Corresponding author. Tel.: +81 75 751 4119; fax: +81 75 751 4646.
E-mail address: iwata@frontier.kuoyo-u.ac.jp (H. Iwata).

http://dx.doi.org/10.1016/j.bbagen.2014.09.025
0304-4165/

patient, the reprogramming protocols may affect the immunogenicity of the grafts [20,21]. In addition, approximately 100 iPS cell lines with human leukocyte antigen (HLA) homozygotes [22] are planning to be established and banked. Due to economic reasons, these cells will be used as sources for cell preparations for patient treatments. Third, although a small number of DA neurons are sufficient to demonstrate the efficacy of cell replacement therapy in PD model mice and monkeys, a large number of cells are needed to treat human PD patients. Sufficient numbers of matured DA neurons are not usually collected from cells adhered on dishes, because matured neurons are very fragile and are easily damaged by mechanical and enzymatic stress [23]. Fourth, when a patient will be treated by transplantation, sufficient amounts of DA neurons should be supplied in a timely fashion, requiring a method of preservation for DA neurons.

Encapsulation technique was widely studied for the preparation of many types of cells in vitro [24]. Some researchers reported that pluripotent stem cells differentiated into DA neurons in alginate microcapsules [25] or synthetic polymer hydrogel [26]. However, the behavior of DA neurons in long-term culture has not been well characterized. We have been evaluating the microencapsulation of islets of Langerhans in agarose microbeads, which effectively protects the cells from rejection in the transplantation model and from mechanical stress [27,28]. In this study, we examined the utility of this cell encapsulation technique for overcoming the abovementioned difficulties with applying DA neurons to PD patients.

2. Methods

2.1. Human iPS (hiPS) cell culture and differentiation into DA neurons

Three lines of hiPS cells (201B7 line [29] and 253G1 line [30], RIKEN Cell Bank, Ibaraki, Japan. Squeaky line, JCRB Cell Bank, National Institute of Biomedical Innovation, Osaka, Japan) were used in this study. 201B7 and 253G1 lines were derived from dermal fibroblasts of the same patient. Squeaky line was derived from human fetus lung cells (MRC-5). All experiments were performed in accordance with ethical guidelines of our university and the cell banks. Undifferentiated hiPS cells were maintained on SNL 76/7 cells (ECACC, Salisbury, UK) as a feeder layer, as previously described [31]. hiPS cells were differentiated into DA-releasing cells via a previously reported method with some modifications [10]. Details of the differentiation culture are shown in Fig. 1. Undifferentiated hiPS cells were seeded on Matrigel (BD Bioscience, San Jose, CA, USA)-coated cell culture dishes. Cells were cultured in DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2.5 mM GlutaMax, 15% KnockOut Serum Replacement (Invitrogen), and 0.1 mM 2-mercaptoethanol (Nacalai Tesque, Inc., Kyoto, Japan) at 37 °C under 5% CO_2. Ten micromolar SB 431542 (Wako Pure Chemical Industries, Osaka, Japan.), 100 nM LDN193189 (Wako), 3 μM CHIR99021 (Wako), 100 ng/mL sonic hedgehog N-terminus (R&D Systems, Minneapolis, MN, USA), 2 mM purmorphamine (Wako), and 100 ng/mL fibroblast growth factor-8 (Wako) were added to culture medium in a time-dependent fashion. KnockOut Serum Replacement was gradually shifted to N2 supplement (Invitrogen) from day 5 to day 11. On day 11, the culture medium was changed to DMEM/F12 supplemented with 2.5 mM GlutaMax, 2% B27 supplement (Invitrogen), 10 ng/mL brain-derived neurotropconhic factor (BDNF, Wako), 10 ng/mL glial cell line-derived neurotrophic factor (GDNF, Wako), 0.2 mM ascorbic acid (Nacalai Tesque), 0.5 mM dibutyryl cyclic adenosine monophosphate (dcAMP, Nacalai Tesque), 1 ng/mL transforming growth factor β3 (TGF-β3, R&D Systems), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were cultured for an additional week for the maturation of neuronal cells. On day 18, subpopulations of the cells were used for the following experiments. The remaining cells were subcultured on a laminin (50 μg/mL)/poly-L-ornithine (50 μg/mL) (LM/PLO)-coated dish for further maturation.

2.2. Formation of cell aggregates

On day 18, cells were detached from culture dishes by treatment with Accumax (Innovative Cell Technologies, Inc., San Diego, CA, USA) for 3 min. Cells were suspended in DMEM/F12 containing 2.5 mM GlutaMax, 2% B27 supplement, 10 ng/mL BDNF, 10 ng/mL GDNF, 0.2 mM ascorbic acid, 0.5 mM dcAMP, 1 ng/mL TGF-β3, 100 U/mL penicillin, and 100 μg/mL streptomycin. U-bottom 96-well plates (BD Falcon, San Jose, CA, USA) were pre-coated with 2% pluronic F127 overnight to inhibit cell adhesion. After washing with phosphate-buffered saline (PBS) five times, the cells were seeded into the wells at a density of 6000 cells/well and centrifuged at 1000 rpm for 3 min. The cells were cultured in an incubator at 37 °C in a 5% CO_2 atmosphere for 5 days to induce the formation of cell aggregates.

2.3. Agarose microencapsulation

Cell aggregates were enclosed in agarose microbeads as previously reported [24]. Briefly, approximately 2000 cell aggregates were mixed with 3 mL of 5% agarose (Taiyo Agarose, AG LT-600, Shimizu Shokuhin KK, Shizuoka, Japan)/PBS at 40 °C in a glass centrifuge tube. Then, 15 mL of liquid paraffin (Nacali Tesque) at 40 °C were added to the centrifuge tube. The tube was shaken on ice to form small droplets of the agarose solution and to induce gelation. The microbead suspension was centrifuged at 2000 rpm for 5 min, and the liquid paraffin was removed. The suspension was washed twice with PBS, and the microbeads containing cell aggregates were selected under a microscope. Encapsulated cells were suspended in DMEM/F12 containing 2.5 mM GlutaMax, 2% B27 supplement, 10 ng/mL BDNF, 10 ng/mL GDNF, 0.2 mM ascorbic acid, 0.5 mM dcAMP, 1 ng/mL TGF-β3, 100 U/mL penicillin, and 100 μg/mL streptomycin. Culture medium was replaced with fresh medium every three days. Encapsulated cells were cultured in a static condition for 45 days.

2.4. Cell viability assay

The live/dead assay was performed by staining cells with calcein-AM and propidium iodide (PI). Cell aggregates were exposed to medium containing 1 μg/mL calcein-AM and 2 μg/mL PI for 1 h and then washed twice with PBS. The fluorescent microphotographs of cells were recorded using a confocal microscope (Fluoview FV10i, Olympus Optical Co., Ltd., Tokyo, Japan).

2.5. DA secretion and high-performance liquid chromatography (HPLC)

Microencapsulated cell aggregates (1200) were washed twice with PBS supplemented with 0.33 mM Mg^{2+} and 0.9 mM Ca^{2+}. The cells were incubated for 30 min in 56 mM KCl/Hanks' balanced salt solution (HBSS) supplemented with 0.33 mM Mg^{2+} and 0.9 mM Ca^{2+} to induce depolarization of cells. The supernatant was collected, and 0.1 mM ethylenediamine-N,N,N′,N′-tetraacetic acid (EDTA) and 0.1 M perchloric acid were added to the supernatant to inhibit DA degradation. The same number of non-encapsulated cell aggregates cultured on LM/PLO was used as a control.

A TSK-GEL Super-ODS column (100 × 4.6 mm; TOSOH, Tokyo, Japan) and an EC8020 electrochemical detector (TOSOH) were used for HPLC of the supernatant. The mobile phase was composed of 0.1 M citrate buffer solution (pH 2.5), 0.1 mM EDTA, 5 mM sodium 1-octanesulfonate, and 3% (v/v) methanol. The flow rate of the mobile phase was 1.2 mL/min.

2.6. Immunofluorescence

Antibodies against octamer-binding transcription factor 3/4 (Oct3/4, 1:50, rabbit polyclonal, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), stage-specific embryonic antigen-4 (SSEA-4, 1:200, mouse monoclonal, Merck Millipore, Billerica, MA, USA), Nanog (1:200, rabbit monoclocal, Cell Signaling Technologies, Inc., MA, USA), Tra-1-81

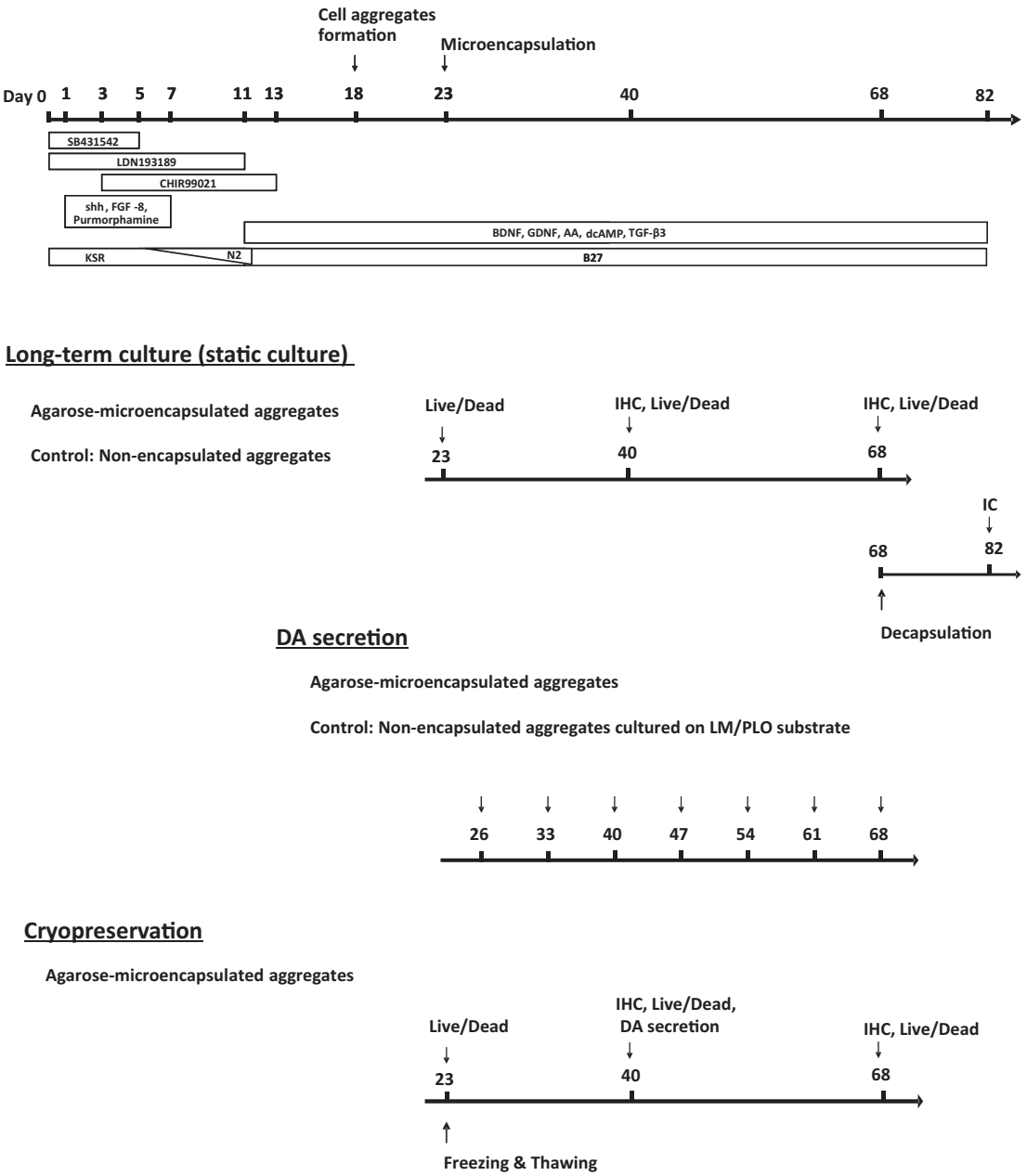

Fig. 1. Experimental protocol. hiPS cells were differentiated into DA neurons in culture medium supplemented with several inhibitors and cytokines. Cells were collected from a dish and applied to U-bottom 96-well plates at a density of 6000 cells/well to form cell aggregates on day 18. The cell aggregates were encapsulated in agarose microbeads on day 23. Microencapsulated aggregates were cultured in a static condition for 45 days.

(1:200,mouse monoclocal, Cell Signaling Technologies), β-tubulin III (1:500, rabbit monoclonal, Covance, Princeton, NJ, USA), tyrosine hydroxylase (TH, 1:200, mouse monoclonal, Millipore, and 1:200, mouse monoclonal, Covance), forkhead box protein A2 (FoxA2, 1:100, goat polyclonal, Santa Cruz), LIM homeobox transcription factor 1 alpha (Lmx1a, 1:1000, rabbit polyclonal, Millipore), nuclear receptor related 1 protein (Nurr1, 1:100, rabbit polyclonal, Santa Cruz) and glial fibrillary acidic protein (GFAP, 1:200, mouse monoclonal, Millipore) were used for immunohistochemistry. Cells were fixed with 4% paraformaldehyde in PBS for 1 h at room temperature, and then sequentially soaked in 5% sucrose/PBS for 12 h at 4 °C, 10% sucrose/PBS for 12 h at 4 °C, and 20% sucrose/PBS for 12 h at 4 °C. The cells were embedded in Tissue-Tek (Sakura Finetechnical Co. Ltd., Tokyo, Japan) and frozen. Frozen specimens of 8 μm thickness were prepared.

The specimens were treated with 0.2% Triton X-100 solution for 15 min at room temperature to permeabilize the cells, and were treated with Blocking One Reagent (Nacarai Tesque) for 90 min to block nonspecific adsorption of antibodies. Antibody solutions were applied to the specimens and incubated for 1 h at room temperature. After washing with PBS containing 0.05% Tween 20, the specimens were treated with Alexa Fluor 594 anti-mouse IgG, Alexa Fluor 488 anti-rabbit IgG, and Alexa Fluor 594 anti-goat IgG (Invitrogen) at a dilution of 1:500 for 1 h at room temperature, then washed with PBS containing 0.05% Tween 20. The cell nuclei were counterstained with 1 μg/mL Hoechst 33258 (Dojindo Laboratories, Kumamoto, Japan). The localization of secondary antibodies was analyzed with a fluorescent microscope (BX51 TRF, Olympus Optical Co., Ltd., Tokyo, Japan). To determine the ratio of TH-positive cells, cells were carefully identified on merged images enlarged with computer software. Cells were counted at three sites on the same sample, and these data were averaged. At least a thousand of cells were counted on each experiment. Data are shown as mean ± standard deviation (sd) for three independent experiments.

2.7. Reverse transcription polymerase chain reaction (RT-PCR)

On days 25, 40, and 68 of culture, encapsulated cells were collected into a centrifuge tube and washed with cold PBS. The microcapsules were homogenized on ice with a sonicator (VP-30S, Titec, Saitama, Japan). Total RNA was extracted with the SV Total RNA Isolation System (Promega Corp., Madison, WI, USA). First-strand cDNA was prepared from the RNA by reverse transcription using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) primed by oligo$(dT)_{18}$. cDNA was then amplified by PCR using the following specific primers [32]: Oct 3/4, 5′-ATT CAG CCA AAC GAC CAT CT-3′ and 5′-ACA CTC GGA CCA CAT CCT TC-3′; Nanog, 5′-AGC ATC CGA CTG TAA AGA ATC TTC AC-3′ and 5′-CGG CCA GTT GTT TTT CTG CCA CCT-3′; E-cadherin, 5′-CGA CCC AAC CCA AGA ATC TA-3′ and 5′-AGG CTG TGC CTT CCT ACA GA-3′; tubulin βIII, 5′-ACC TCA ACC ACC TGG TAT CG-3′ and 5′-TGC TGT TCT TGC TCT GGA TG-3′; TH, 5′-GAG TAC ACC GCC GAG GAG ATT G-3′ and 5′-GCG GAT ATA CTG GGT GCA CTG G-3′; Nurr1, 5′-CTC CCA GAG GGA ACT GCA CTT CG-3′ and 5′-CTC TGG AGT TAA GAA ATC GGA GCT G-3′; Lmx1b, 5′-GCA GCG GCT GCA TGG AGA AGA TCG C-3′ and 5′-GGT TCT GAA ACC AGA CCT GGA CCA C-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-ACC ACA GTC CAT GCC ATC AC-3′ and 5′-TCC ACC ACC CTG TTG CTG TA-3′. The reaction mixtures (20 μL) containing 1 μL cDNA template, 5U Takara Ex Taq DNA polymerase (Takara Bio Inc., Shiga, Japan), 1 μM sense primer, and 1 μM antisense primer were subjected to PCR under the following thermal cycling conditions: denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s. PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining.

2.8. Collection of cell aggregates from microbeads and adherent culture

Agarose microbeads were mechanically disrupted with a scalpel under a microscope to collect cell aggregates. The cell aggregates were seeded on a LM/PLO-coated dish and cultured in DMEM/F12 containing 2.5 mM GlutaMax, 2% B27 supplement, 10 ng/mL BDNF, 10 ng/mL GDNF, 0.2 mM ascorbic acid, 0.5 mM dcAMP, 1 ng/mL TGF-β3, 100 U/mL penicillin, and 100 μg/mL streptomycin. After 14 days of culture, the cells were fixed with paraformaldehyde and immunologically stained as the same way of Section 2.6.

2.9. Function of cell aggregates in microbeads after cryopreservation

Cell aggregates in agarose microbeads were cryopreserved as previously reported [18]. Chilled KYO-1 solution (5.38 M ethylene glycol, 2 M dimethyl sulfoxide, 0.1 M polyethylene glycol 1000, and 0.00175 M polyvinylpyrrolidone (molecular weight 10,000) in Euro-Collins solution) was used for vitrification. Briefly, on day 23, 800 cell aggregates in agarose microbeads were suspended in 100 μL of Euro-Collins solution in a cryotube. Chilled KYO-1 solution was sequentially added and the suspension was incubated as follows: addition of 10 μL KYO-1 and incubation for 10 min at room temperature, addition of 5 μL KYO-1 and incubation for 15 min at room temperature, addition of 15 μL KYO-1 and incubation for 15 min on ice, and addition of 980 μL KYO-1

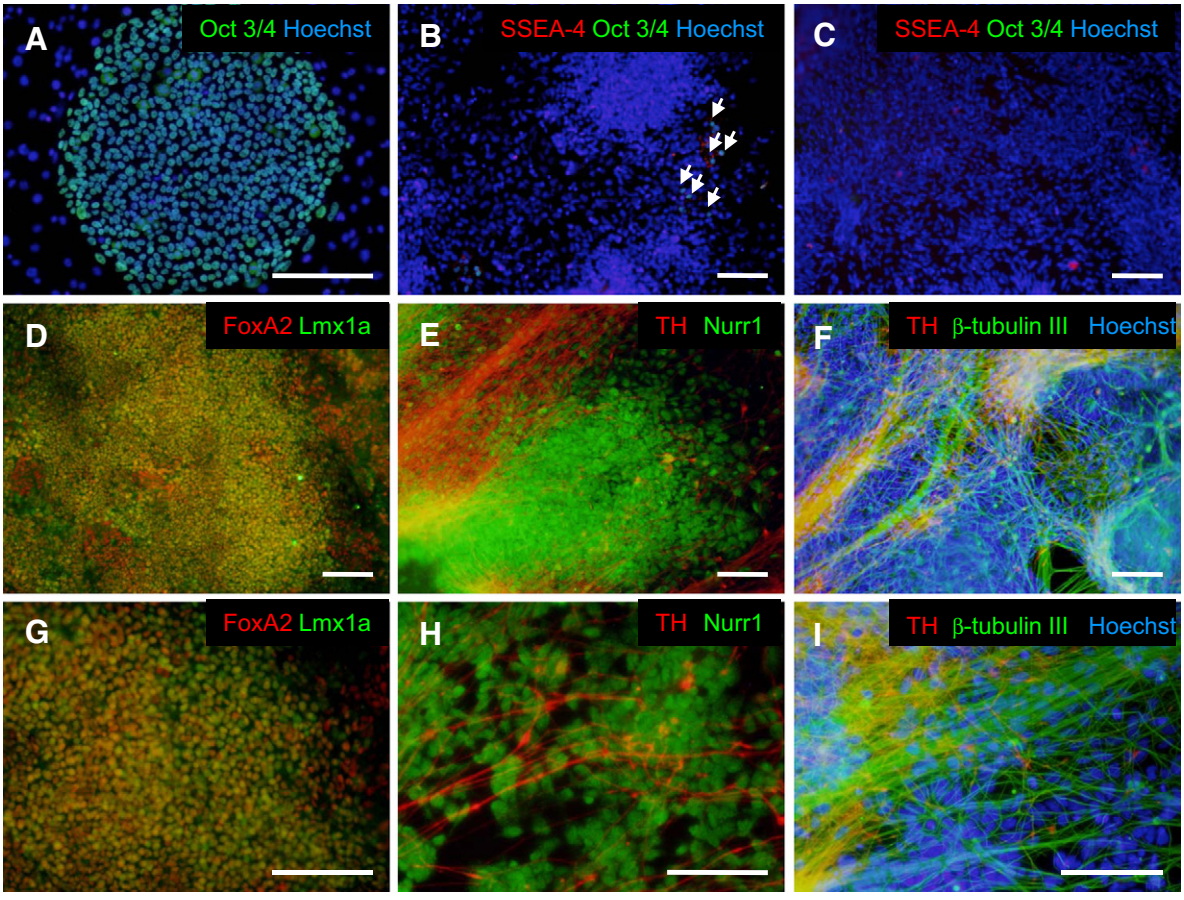

Fig. 2. Differentiation of hiPS cells into DA neurons (253G1 line). A: Fluorescent micrograph of undifferentiated hiPS cells on SNL feeder cells. The cells were immunologically stained with an antibody against Oct 3/4, and nuclei were stained with Hoechst 33258. B, C: Fluorescent micrographs of cells on day 11 (B) and on day 18 (C). Cells were immunologically stained with antibodies against Oct 3/4 and SSEA-4, and nuclei were stained with Hoechst 33258. Arrows indicate Oct 3/4 positive cells. D–F: Fluorescent micrographs of cells on day 18. Cells were immunologically stained with antibodies against FoxA2 and Lmx1A (D), TH and Nurr1 (E), TH and β-tubulin III (F). G–I: High magnification images of D–F. Scale bar: 100 μm.

and incubation for 5 min at 0 °C. After these procedures, the cryotube was quickly immersed in liquid nitrogen for 3 min, and then stored in the vapor phase of liquid nitrogen. After 24 h of cryopreservation, the cryotube was immersed into a 30% dimethyl sulfoxide-water solution at room temperature to thaw the suspension rapidly. The suspension was transferred to a conical tube containing 10 mL Euro-Collins solution and centrifuged at 1000 rpm for 3 min. The supernatant was discarded and the microbeads were resuspended in 0.75 M sucrose solution (DMEM/F12, 2% B27 supplement) and incubated at 0 °C for 30 min to remove intracellular vitrification solution. Then, ice-cold culture medium (1 mL DMEM/F12 plus 2% B27 supplement) was added every 5 min for 20 min. The final suspension was centrifuged at 1000 rpm for 3 min and resuspended in culture medium. Cells were cultured for 17 days for maturation. Seven hundred cell aggregates in microbeads were used for the analysis of DA secretion.

2.10. Statistical analysis

Data are shown as mean ± standard deviation (sd) for at least three independent experiments. The data were compared by Student's *t*-test. All statistical calculations were performed by using the software JMP (SAS Institute Inc., NC, USA).

3. Results

3.1. Differentiation of hiPS cells into DA neurons

hiPS 201B7 line and 253G1 line were mainly used in this study. The cell lines were derived from the same patient. Dual-SMAD inhibition and floor-plate induction protocol [10], which was developed for effective differentiation of human ES cells into midbrain DA neurons, was used to induce differentiation of hiPS cells into DA neurons with some modification (Fig. 1). Briefly, undifferentiated hiPS cells were seeded on a Matrigel-coated dish and cultured in medium containing inhibitors and cytokines for 18 days. Morphological changes in the iPS cells were observed after a few days in culture. Expression of pluripotent markers such as SSEA-4 and Oct 3/4 rapidly decreased and was hardly detectable at day 18 by immunohistochemistry (Fig. 2A–C). On day 18, 60–80% of cells expressed midbrain DA neuron markers such as FoxA2, Lmx1a, Nurr1 (Fig. 2D–E, G–H). At the time, half of the cells were positive for β-tubulin III, a marker of both immature and mature neurons, but most cells were negative for TH, a marker of DA neurons (Fig. 2F and I). Progenitor cells and immature neurons coexisted in the culture. The cells were subcultured onto LM/PLO substrate and cultured for an additional week to induce lineage-specific differentiation. Approximately 30% of the cells became TH-positive by day 25 (Fig. S1A). These observations indicate that the hiPS cells were successfully differentiated into the DA neuron lineage. On day 50, cells also expressed dopamine neuron markers (Fig. S1B and S1C).

3.2. Agarose microencapsulation

On day 18 of culture, cells were collected from the culture dishes and seeded into U-bottom 96-well plates pre-coated with pluronic F127 at a density of 6000 cells/well to induce cell aggregate formation. After 5 days, a cell aggregate with diameter of 300–400 μm was found in each well. The cell aggregates were collected from the plates and microencapsulated in agarose microbeads as previously reported [27]. Fig. 3 contains phase contrast micrographs of microencapsulated cell aggregates and images of hematoxylin and eosin staining of thin sections before and immediately after microencapsulation. Cells exhibited a compact multicellular morphology even after encapsulation (Fig. 3E and F). Live/Dead assay revealed the most of cells were living after microencapsulation (Fig. 3G and H). No clear difference was observed in the percentage of PI-positive cells between encapsulated cells and non-encapsulated cells.

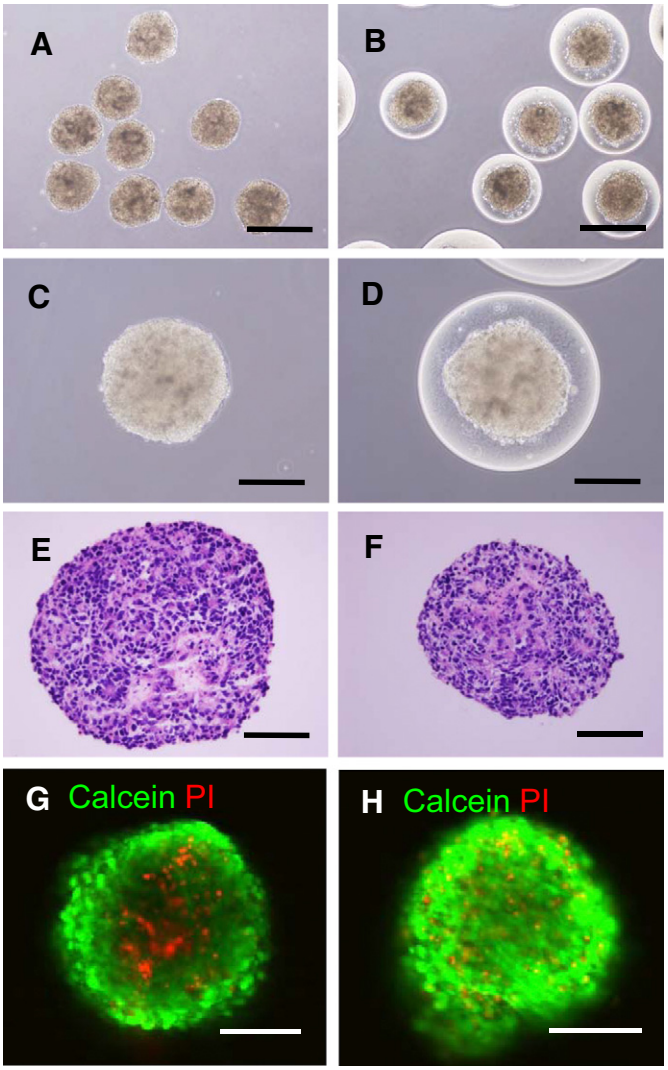

Fig. 3. Effect of microencapsulation on the morphology of cell aggregates derived from hiPS cells (253G1 line). A–D: Phase contrast micrographs of cell aggregates before (A, C) and after (B, D) microencapsulation. E, F: Hematoxylin and eosin staining of cell aggregates before (E) and after (F) microencapsulation. G, H: Confocal images of cell aggregates before (G) and after (H) microencapsulation. Cells were stained with calcein-AM (green) and PI (red). Scale bars: 500 μm (A, B), 200 μm (C, D), and 100 μm (E–H).

3.3. Long-term culture

Cell aggregates were cultured for an additional 45 days to induce maturation of DA neurons. Free cell aggregates that lacked microencapsulation gathered together and formed large aggregates (Fig. 4A–C and E–G). The core of the aggregates became dark. Cell necrosis was seen in the core of an aggregate due to limited supplies of oxygen and nutrients as indicated by considerable number of PI-positive cells (Fig. 4D and H). TH-positive cells observed only in periphery of the aggregates (Fig. 4I–P).

When the cell aggregates were microencapsulated, formation of large aggregates was inhibited and cells located at the center of the aggregates were still living after long-term culture (Fig. 5A–H). Fig. 5I–P contains immunofluorescent images of cell aggregates in microbeads. Most of the cells were tubulin βIII-positive, and some cells became TH-positive on days 40 and 68. The percentage of cells positive for both tubulin βIII and TH were 42.0 ± 8.7% and 66.3 ± 7.1% on days 40 and 68, respectively. Encapsulated cells were also stained with other DA neuron makers such as FoxA2, Lmx1A, Nurr1. GFAP-positive glial cells were hardly detectable at day 40, but a few GFAP-positive cells were observed on day 68 (Fig. S2).

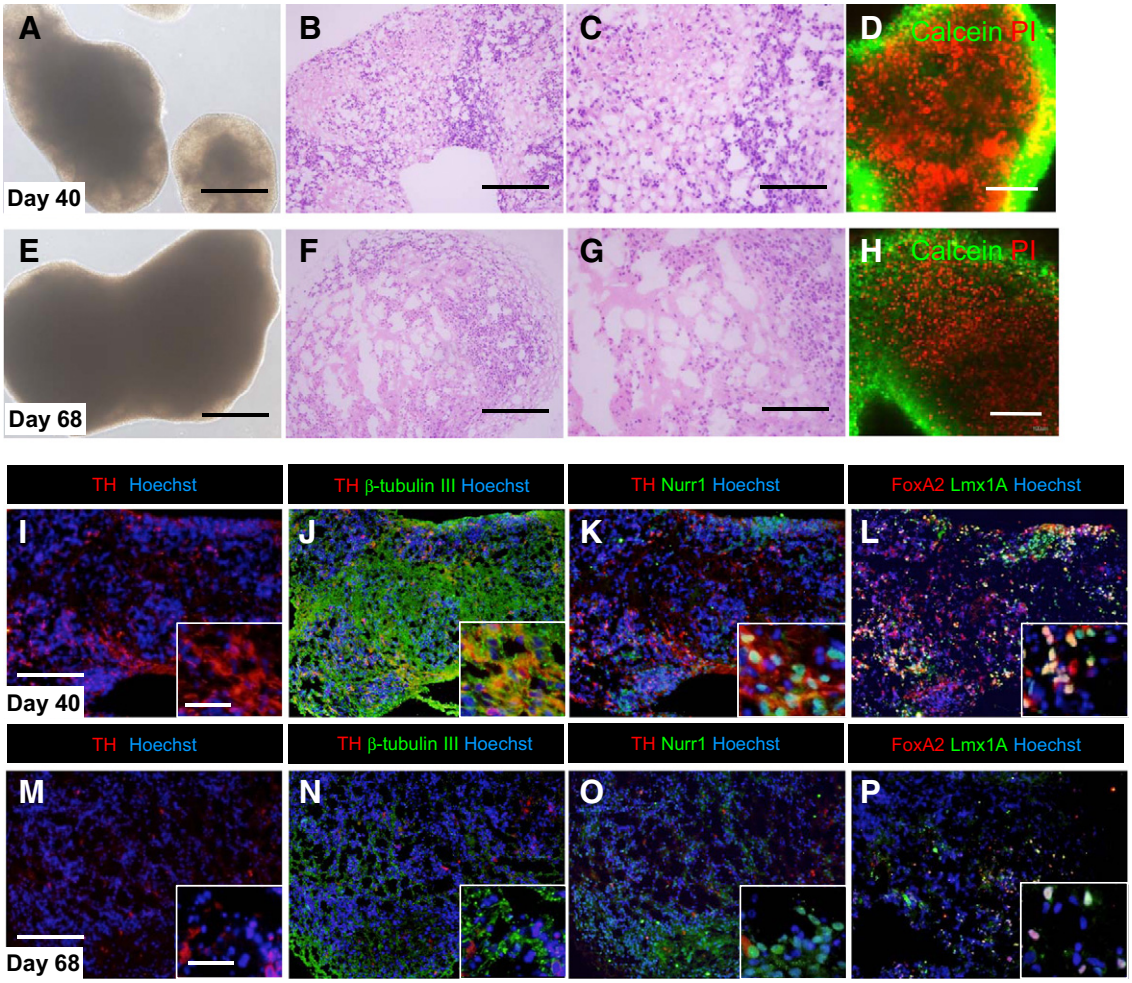

Fig. 4. Long-term culture of free cell aggregates (253G1 line). A, E: Phase contrast microscopy of free cell aggregates on day 40 (A) and day 68 (E). B, C, F, and G: Hematoxylin and eosin staining of thin sections of free cell aggregates on day 40 (B, C) on and day 68 (F, G). D, H: Confocal images of free cell aggregates on day 40 (D) and day 68 (H). Cells were stained with calcein-AM (green) and PI (red). I–P: Fluorescent micrograph of free cell aggregates on day 40 (I–L) and day 68 (M–P). Cells were immunologically stained with antibodies against TH (I, M), TH and β-tubulin III (J, N), TH and Nurr1 (K, O), FoxA2 and Lmx1A (K, P). Cell nuclei were stained with Hoechst 33258. Scale bars: 500 μm (A, B, E, F), 200 μm (C, D, G, H, I–P), and 50 μm (high magnification images in I–P).

RT-PCR of these cell aggregates revealed the expression of tubulin βIII and TH (Fig. 5R). Expression of other DA neuron markers, such as Nurr1 and Lmx1b, were also detected by RT-PCR (Fig. 5R). These results indicate that the cells were differentiated into DA neurons, which were maintained in the cell aggregates after 68 days of culture. Pluripotent stem cell markers Oct 3/4, Nanog, SSEA-4 and Tra-1-81 were not detected in immunofluorescent images (Fig. S3) of encapsulated aggregates on day 40 and day 68. RT-PCR, however, indicated that expression of Oct 3/4 disappeared at day 25, but were slightly positive on day 40 and day 68 (Fig. 5R). Cell aggregates derived from hiPS Squeaky line also could be maintained in agarose microbeads (Fig. S4). The encapsulated cells were stained with DA neuron markers such as TH and Nurr1 (Fig. S4C-D) and produced an adequate amount of dopamine (Fig. S4E). Agarose beads were mechanically disrupted on day 68 to collect cell aggregates, which were seeded onto LM/PLO substrate. Many neurites extended from the aggregates within 1 day, and were elongated over 1 mm after 2 weeks (Fig. 6).

3.4. DA production

DA production by cells in microbeads was studied by depolarization in 56 mM KCl. Twelve hundred cell aggregates were incubated for 30 min in 0.5 mL of 56 mM KCl in HBSS. The supernatants were collected and analyzed by HPLC (Fig. 7A). The DA peak was small on day 26, but became large and clearly visible after 61 days of cell culture (Fig. 7A). The amounts of DA produced by the microencapsulated cells gradually increased during culture. DA production was sustained to the end of culture (Fig. 7B). The low level of DA production at day 26 was due to insufficient maturation of cells, and coincided with a small ratio of TH-positive cells in the cell aggregates at day 26.

DA production by cell aggregates that were cultured on LM/PLO substrate (Fig. S5) was also examined to assess the effects of microencapsulation on DA production. DA production by cells in adherent culture gradually increased throughout the culture period. The amounts of production were similar to those of the encapsulated cells (Fig. 7B). No statistical difference in DA production was observed between cells on LM/PLO substrate and cells in agarose beads.

3.5. Cryopreservation of encapsulated cell aggregates

Vitrification was employed for cryopreservation of cell aggregates in agarose microbeads to avoid intracellular ice formation and to preserve the integrity of agarose capsules [18]. Cell aggregates from day 23 were used for cryopreservation. Microbeads and cell aggregates exhibited no

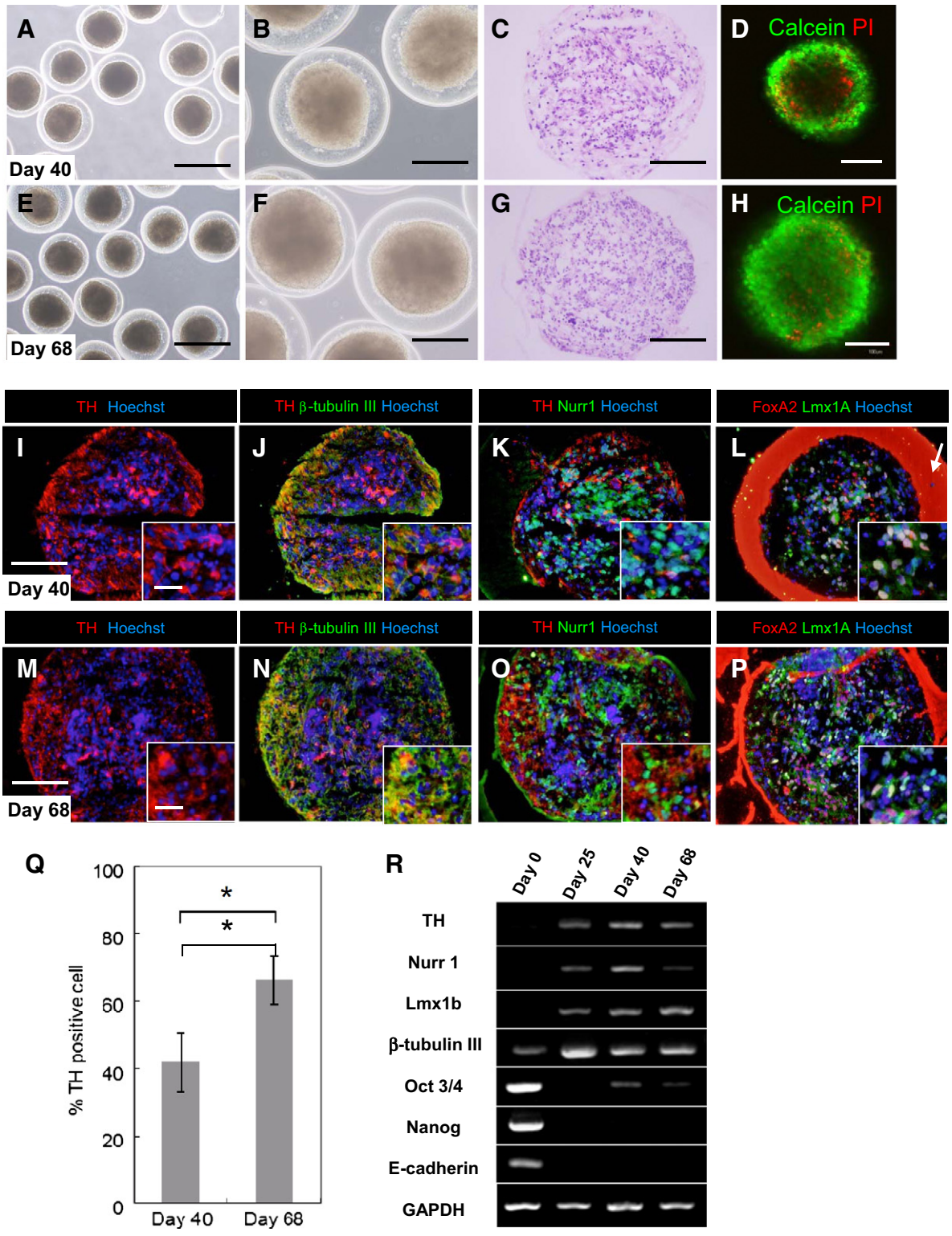

Fig. 5. Long-term culture of microencapsulated DA neurons (253G1 line). A, B, E, F: Phase contrast microscopy of microencapsulated cell aggregates on day 40 (A, B) and day 68 (E, F). C, G: Hematoxylin and eosin staining of thin sections of microencapsulated cell aggregate on day 40 (C) on and day 68 (G). D, H: Confocal images of free cell aggregates on day 40 (D) and day 68 (H). Cells were stained with calcein-AM (green) and PI (red). I–P: Fluorescent micrograph of microencapsulated cell aggregates on day 40 (I–L) and day 68 (M–P). Cells were immunologically stained with antibodies against TH (I, M), TH and β-tubulin III (J, N), TH and Nurr1 (K, O), FoxA2 and Lmx1A (K, P). Cell nuclei were stained with Hoechst 33258. Scale bars: 500 μm (A, E), 200 μm (B, F), 100 μm (C, D, G, H, I–P) and 50 μm (high magnification images in I–P). Q: Percentage of TH-positive cells in cell aggregates on day 40 and day 68 (mean ± sd., n = 3). In each experiment, three aggregates were analyzed. The experiments were repeated three times. An asterisk indicates statistical significance (Student's-*t* test, $p < 0.05$). R: RT-PCR of cells on day 0, day 25, day 40, and day 68.

morphological differences before and after freezing and thawing (Fig. 8A, D). These cells were further cultured to induce maturation. After 17 days, no morphological changes or cell death in the cell aggregates were observed (Fig. 8B, E). TH-positive cells were observed on day 40 (Fig.8I, K). The amounts of DA secreted from cryopreserved cells were the same as those secreted by non-cryopreserved cells

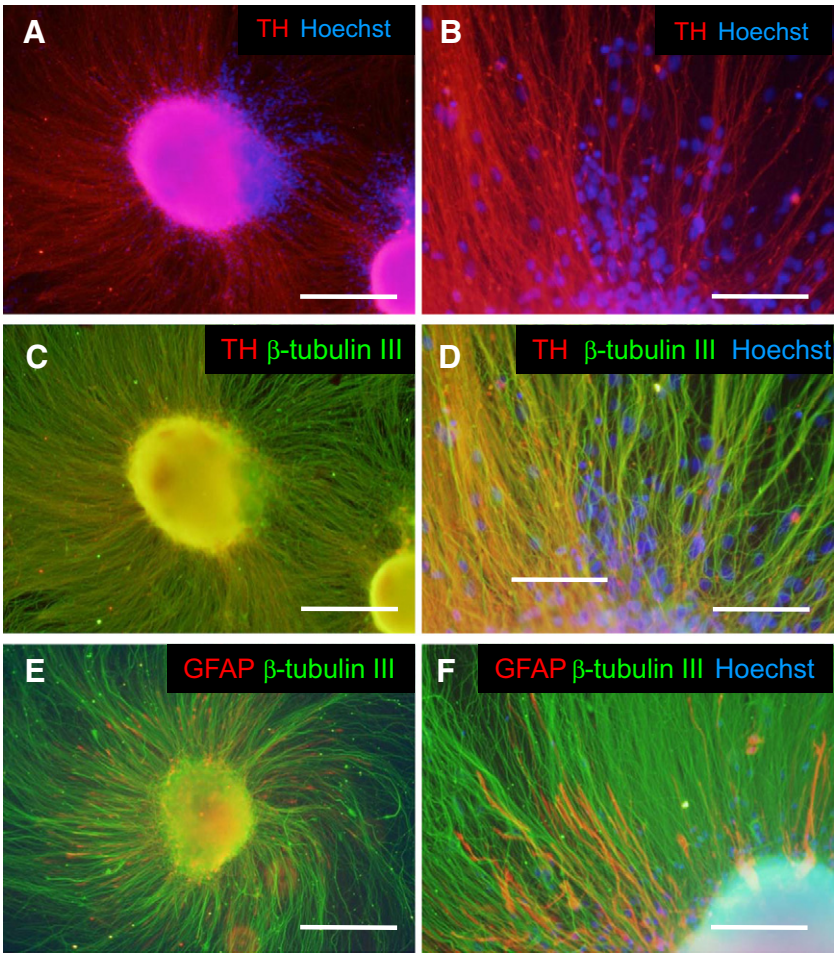

Fig. 6. Potential of cells in aggregates to extend neurites. Agarose microbeads were mechanically disrupted to collect cell aggregates on day 68, and the cell aggregates were cultured on LM/PLO substrate for 14 days. A–D: Fluorescent micrograph of cells immunologically stained using antibodies against TH (red) and β-tubulin III (green). E, F: Fluorescent micrograph of cells immunologically stained using antibodies against GFAP (red) and β-tubulin III (green). Cell nuclei were stained with Hoechst 33258. Scale bars: 500 μm (A, C, E) and 100 μm (B, D, F).

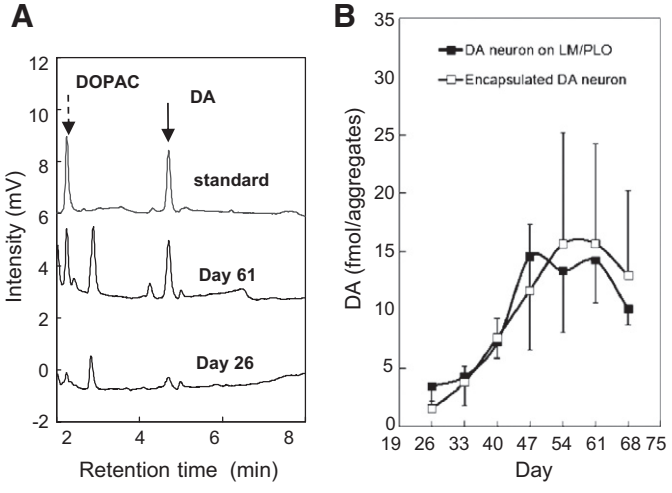

Fig. 7. DA production by cell aggregates in agarose microbeads. Microencapsulated cell aggregates (1200) were depolarized in 56 mM KCl for 30 min. The supernatants were analyzed by reverse-phase HPLC. A: Representative HPLC output: upper line, standard solution of 20 nM DA and 20 nM DOPAC; middle line, supernatant from microencapsulated cell aggregates on day 68; bottom line, supernatant from microencapsulated cell aggregates on day 26. Solid and dashed arrows indicate the peaks for DA and DOPAC, respectively. B: Temporal changes in DA production levels (mean ± sd.). Black and white boxes indicate the levels of cell aggregates cultured on LM/PLO substrate (n = 3) and microencapsulated cell aggregates (n = 5).

(Fig. 8M). After long-term culture, the cells were still living and expressed DA neuron makers (Fig. 8C, F, J).

4. Discussion

The floor-plate induction protocol [10] was employed to induce differentiation of iPS cells into DA neurons. On day 18, cells were detached from culture dishes and applied to U-bottom 96-well plates to form cell aggregates. The cell aggregates were enclosed into agarose microbeads and cultured for differentiation into DA neurons. Approximately 66% of all cells became DA neurons. Although LM/PLO substrate was employed to mature cells into DA neurons in the original protocol, hiPS cells in aggregates efficiently differentiated into DA neurons in agarose microcapsules. Cell aggregates without encapsulation adhered each to other and formed large aggregates, and the amount of DA produced from these aggregates was much smaller than that produced by the cell aggregates in microbeads due to necrosis in the core of the large cell aggregates. Agarose microbeads effectively inhibited formation of large cell aggregates and did not disrupt the differentiation of hiPS cells into DA neurons. No clear difference in DA production was observed between cells on LM/PLO substrate and cells encapsulated in agarose beads (Fig. 7). DA from DA neurons rapidly passed through the agarose layer of the microbeads and was released into the medium, suggesting that the microbeads did not hinder DA release. Additionally, DA neurons in the microbeads could be handled without specific protocols, since the microbeads protected the fragile DA neurons from mechanical stress.

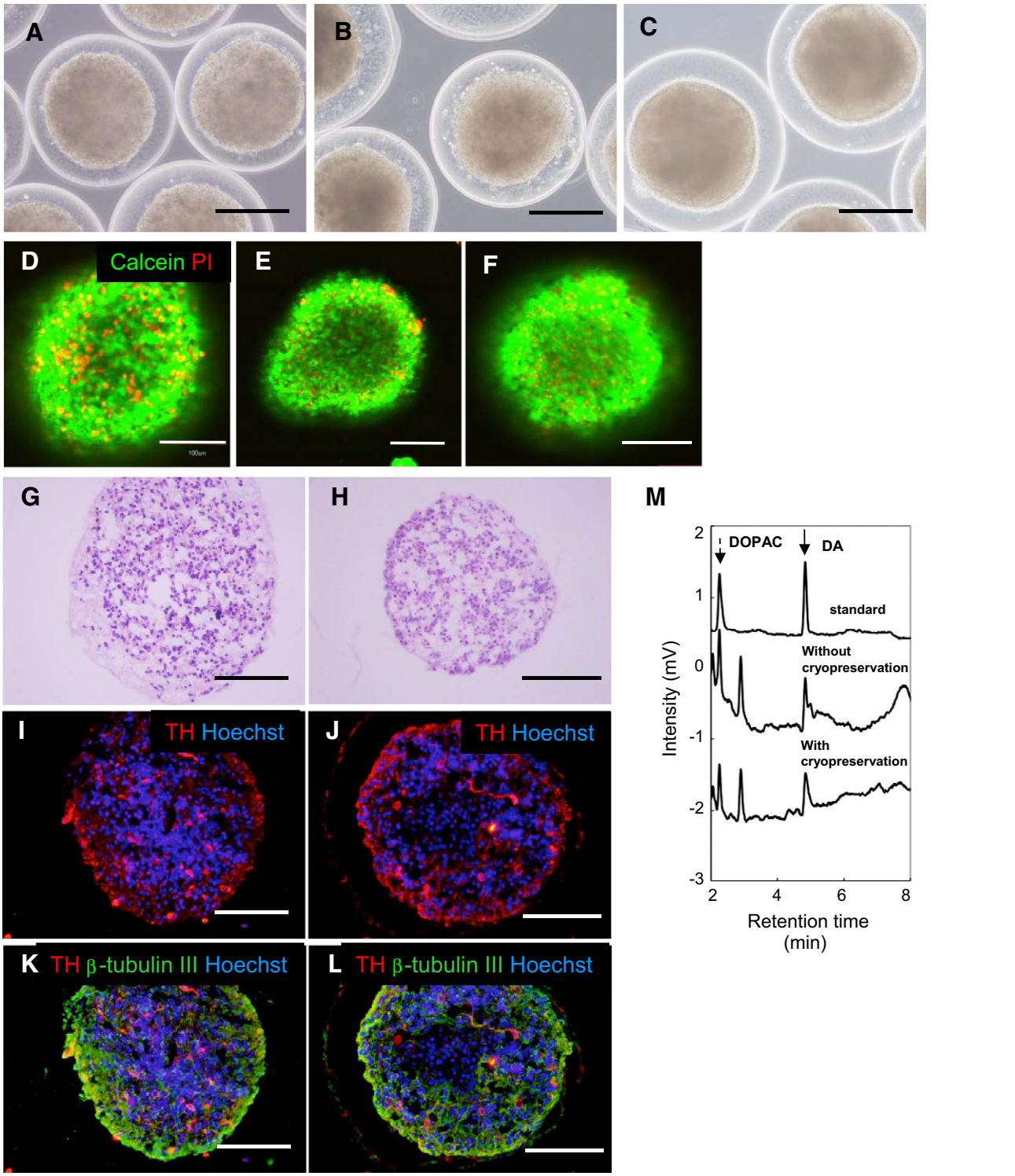

Fig. 8. Cryopreservation of cell aggregates (253G1 line) in agarose microbeads. A–C: Phase contrast microscopy of cryopreserved cell aggregates immediately after thawing (A), on day 40 (B), and on day 68 (C). C–F: Confocal images of cryopreserved cells immediately after thawing (D), on day 40 (E), and on day 68 (F). Cells were stained with calcein-AM (green) and PI (red). G, H: Hematoxylin and eosin staining of thin sections of cell aggregates on day 40 (G) and on day 68 (H). I–L: Fluorescent micrograph of cells on day 40 (I, K) and on day 68 (J, L) immunologically stained with antibodies against TH (red) and β-tubulin III (green). Cell nuclei were stained with Hoechst 33258. Scale bars: 200 μm (A–C) and 100 μm (B–J). M: HPLC chromatograms of supernatants from depolarization studies of the cell aggregates in 56 mM KCl for 30 min on day 40. Seven hundred cell aggregates in microbeads were used for the analysis of DA secretion. Representative HPLC output: upper line, standard solution of 10 nM DA and 10 nM DOPAC; middle line, supernatant from microencapsulated cell aggregates with cryopreservation; bottom line, supernatant from microencapsulated cell aggregates without cryopreservation. Solid and dashed arrows indicate the peaks for DA and DOPAC, respectively.

Once cells have been encapsulated, it is relatively easy to maintain them in large quantities. More than 1000 capsules can be cultured in a flask without the need for special protocols. Cell aggregates in microbeads can be cryopreserved (Fig. 8). DA-releasing cells should be supplied at the time of patient transplantation. Therefore, cryopreservation of DA neurons is one way to realize this goal. Attention should be paid to graft volume, as there is limited space in brain. When DA neurons are enclosed into microbeads, the volume of the graft will become several times larger

than that of cells without microencapsulation. It was reported that more than 100,000 surviving DA neurons in each putamen are required for the treatment of PD [9]. In the present study, each microbead contained 3×10^3–4×10^3 DA neurons, as estimated from the number of TH-positive cells (Fig. 5) and from the measurements of DA release (Fig. 7). Approximately 25–40 microbeads are required to treat a human patient, for a total graft volume of less than 0.01 mL. It will not be difficult to transplant such a small volume of microbeads. Taken together, these properties indicate that agarose microbeads are suitable for preparing a large number of DA neurons for treatment of human PD patients.

Tumor formation is a substantial problem during cell transplantation therapy using iPS derived-cells [13,14]. Undifferentiated pluripotent stem cells and neural progenitors contained in transplants may proliferate and overgrow in the host brain [15]. We expect that the risk of tumor formation can be reduced by maturing cells for a long period in vitro before transplantation, as previously reported [16,17]. However, our RT-PCR analyses indicated that expression of Oct 3/4 disappeared at day 25, but was again detectable at day 40 and day 68 (Fig. 5R). We are uncertain as to the significance of the reoccurrence of Oct 3/4 expression at this point. Another pluripotent stem cell marker, Nanog, was not detected by RT-PCR after differentiation culture. The pluripotent stem cell markers such as Oct 3/4, Nanog, SSEA-4 and Tra-1-81 were not detected in immunofluorescent images (Fig. S3). Presence of undifferentiated cells should be carefully examined.

Graft rejection by the host immune system is unavoidable in transplantation therapy. DA neurons derived from ES cells are expected to be recognized as allogeneic tissue in patients. Reprogramming protocols for the preparation of iPS cells may affect the immunogenicity of iPS cells [18,19]. The host immune system responds to cells derived from these cells [20,21]. Immune reactions against DA neurons from banked iPS cells should be carefully controlled. Given the observations reported here, we expect that agarose microbeads will provide an environment favorable to the survival of DA neurons, as agarose microbeads effectively protect allogeneic islet grafts from rejection in mice diabetes models [27,28].

Some researchers have claimed that DA release in the substantia nigra is not sufficient to treat PD, that DA neurons should be integrated with the host brain, and that DA release is regulated through synaptic connections [9]. If these claims were valid, agarose microcapsulation could become an obstacle. We plan to carry out careful examinations of the functions of microencapsulated DA neurons *in vivo* in future works.

5. Conclusion

hiPS-derived precursors of DA neurons were encapsulated in agarose microbeads. Approximately 66% of all cells differentiated into TH-positive neurons in agarose microbeads. These cells released DA for more than 40 days. In addition, the microbeads containing cells could be cryopreserved. Agarose microencapsulation therefore provides a good supporting environment for the preparation and storage of DA neurons.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Scientific Research on Innovative Areas (No. 23107008), by Scientific Research (A) (No. 24240078), and by Challenging Exploratory Research (No. 23650257) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan. SK was financially supported by the program of the Promotion of Science Fellows (No. 4864) from MEXT.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.09.025.

References

[1] W. Dauer, S. Przedborski, Parkinson's disease: review mechanisms and models, Neuron 39 (2003) 889–909.
[2] J.G. Nurt, Wooten Gf, Diagnosis and initial management of Parkinson's disease, N. Engl. J. Med. 353 (2005) 1021–1027.
[3] R. Cools, Dopaminergic modulation of cognitive function-implications for L-DOPA treatment in Parkinson's disease, Neurosci. Biobehav. 30 (2003) 1–23.
[4] O. Rascol, D.J. Brooks, A.D. Korczyn, P.P. De Deyn, C.E. Clarke, A.E. Lang, A five-year study of the incidence of dyskinesia in patients with early Parkinson's disease who were treated with ropinirole or levodopa, N. Engl. J. Med. 342 (2000) 1484–1491.
[5] C.R. Freed, P.E. Greene, R.E. Breeze, W.Y. Tsai, W. DuMouchel, R. Kao, S. Dillon, H. Winfield, S. Culver, J.Q. Trojanowski, D. Eidelberg, S. Fahn, Transplantation of embryonic dopamine neurons for severe Parkinson's disease, N. Engl. J. Med. 344 (2001) 710–719.
[6] R.A. Hauser, T.B. Freeman, B.J. Snow, M. Nauert, L. Gauger, J.H. Kordower, C.W. Olanow, Long-term evaluation of bilateral fetal nigral transplantation in Parkinson disease, Arch. Neurol. 56 (1999) 179–187.
[7] I. Mendez, R. Sanchez-Pernaute, O. Cooper, A. Viñuela, D. Ferrari, L. Björklund, A. Dagher, O. Isacson, Cell type analysis of functional fetal dopamine cell suspension transplants in the striatum and substantia nigra of patients with Parkinson's disease, Brain 128 (2005) 1498–1510.
[8] D.A. Robinton, G.Q. Daley, The promise of induced pluripotent stem cells in research and therapy, Nature 481 (2012) 295–305.
[9] O. Lindvall, Z. Kokaia, Prospects of stem cell therapy for replacing dopamine neurons in Parkinson's disease, Trends Pharmacol. Sci. 30 (2009) 260–267.
[10] S. Kriks, J.W. Shim, J. Piao, Y.M. Ganat, D.R. Wakeman, Z. Xie, L. Carrillo-Reid, G. Auyeung, C. Antonacci, A. Buch, L. Yang, M.F. Beal, D.J. Surmeier, J.H. Kordower, V. Tabar, L. Studer, Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease, Nature 480 (2011) 547–551.
[11] Y.H. Rhee, J.Y. Ko, M.Y. Chang, S.H. Yi, D. Kim, C.H. Kim, J.W. Shim, A.Y. Jo, B.W. Kim, H. Lee, S.H. Lee, W. Suh, C.H. Park, H.C. Koh, Y.S. Lee, R. Lanza, K.S. Kim, S.H. Lee, Protein-based human iPS cells efficiently generate functional dopamine neurons and can treat a rat model of Parkinson disease, J. Clin. Invest. 121 (2011) 2326–2335.
[12] A. Swistowski, J. Peng, Q. Liu, P. Mali, M.S. Rao, L. Cheng, X. Zeng, Efficient generation of functional dopaminergic neurons from human induced pluripotent stem cells under defined conditions, Stem Cells 28 (2010) 1893–1904.
[13] H. Hentze, R. Graichen, A. Colman, Cell therapy and the safety of embryonic stem cell-derived grafts, Trends Biotechnol. 25 (2010) 24–32.
[14] C.Y. Fong, K. Gauthaman, A. Bongso, Teratomas from pluripotent stem cells: a clinical hurdle, J. Cell. Biochem. 111 (2010) 769–781.
[15] J.Y. Li, N.S. Christophersen, V. Hall, D. Soulet, P. Brundin, Critical issues of clinical human embryonic stem cell therapy for brain repair, Trends Neurosci. 31 (2008) 146–153.
[16] A. Brederlau, A.S. Correia, S.V. Anisimov, M. Elmi, G. Paul, L. Roybon, A. Morizane, F. Bergquist, I. Riebe, U. Nannmark, M. Carta, E. Hanse, J. Takahashi, Y. Sasai, K. Funa, P. Brundin, P.S. Eriksson, J.Y. Li, Transplantation of human embryonic stem cell-derived cells to a rat model Parkinson's disease: effect of in vitro differentiation on graft survival and teratoma formation, Stem Cells 24 (2006) 1433–1440.
[17] C1. Seminatore, J. Polentes, D. Ellman, N. Kozubenko, V. Itier, S. Tine, L. Tritschler, M. Brenot, E. Guidou, J. Blondeau, M. Lhuillier, A. Bugi, L. Aubry, P. Jendelova, E. Sykova, A.L. Perrier, B. Finsen, B. Onteniente, The postischemic environment differentially impacts teratoma or tumor formation after transplantation of human embryonic stem cell-derived neural progenitors, Stroke 41 (2010) 153–159.
[18] M. Drukker, N. Benvenisty, The immunogenicity of human embryonic stem-derived cells, Trends Biotechnol. 22 (2004) 136–141.
[19] J.A. Bradley, E.M. Bolton, R.A. Pedersen, Stem cell medicine encounters the immune system, Nat. Rev. Immunol. 2 (2002) 859–871.
[20] C.J. Taylor, E.M. Bolton, J.A. Bradley, Immunological considerations for embryonic and induced pluripotent stem cell banking, Philos. Trans. R. Soc. Lond. B Biol. Sci. 366 (2011) 2312–2322.
[21] T. Zhao, Z.N. Zhang, Z. Rong, Y. Xu, Immunogenicity of induced pluripotent stem cell, Nature 474 (2011) 212–215.
[22] N. Nakatsuji, F. Nakajima, K. Tokunaga, HLA-haplotype banking and iPS cells, Nat. Biotechnol. 26 (2008) 739–740.
[23] M. Emgard, K. Blomgren, P. Brundin, Characterisation of cell damage and death in embryonic mesencephalic tissue: a study on ultrastructure, vital stains and protease activity, Neuroscience 115 (2002) 1177–1187.
[24] A. Murua, A. Portero, G. Orive, R.M. Hernández, M. de Castro, J.L. Pedraz, Cell microencapsulation technology: towards clinical application, J. Control. Release 132 (2008) 76–83.
[25] J. Kim, P. Sachdev, K. Sidhu, Alginate microcapsule as a 3D platform for the efficient differentiation of human embryonic stem cells to dopamine neurons, Stem Cell Res. 11 (2013) 978–989.
[26] Y. Lei, D.V. Schaffer, A fully defined and scalable 3D culture system for human pluripotent stem cell expansion and differentiation, Proc. Natl. Acad. Sci. 110 (2013) 5039–5048.
[27] H. Iwata, T. Takagi, H. Amemiya, H. Shimizu, K. Yamashita, K. Kobayashi, T. Akutsu, Agarose for a bioartificial pancreas, J. Biomed. Mater. Res. 26 (1992) 967–977.
[28] C.A. Agudelo, Y. Teramura, H. Iwata, Cryopreserved agarose-encapsulated islets as bioartificial pancreas: a feasibility study, Transplantation 87 (2009) 29–34.

[29] K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, S. Yamanaka, Induction of pluripotent stem cells from adult human fibroblasts by defined factors, Cell 131 (2007) 861–872.
[30] M. Nakagawa, M. Koyanagi, K. Tanabe, K. Takahashi, T. Ichisaka, 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.
[31] T. Nishigaki, Y. Teramura, A. Nasu, K. Takada, J. Toguchida, H. Iwata, Highly efficient cryopreservation of human induced pluripotent stem cells using a dimethyl sulfoxide-free solution, Int. J. Dev. Biol. 55 (2011) 305–311.
[32] T. Ando, H. Yamazoe, K. Moriyasu, Y. Ueda, H. Iwata, Induction of dopamine-releasing cells from primate embryonic stem cells enclosed in agarose microcapsules, Tissue Eng. 13 (2007) 2539–2547.