DOSSIER ANNEXES

PROTON

MAGNETIC FREEZING TECHNOLOGY IN **BIOMEDICAL CRYOPRESERVATION**

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Technical and Bibliographic Annexes

Appendix A – Scientific Articles and Key Studies

1. Hiramatsu *et al* ., 2022 (Journal of Parkinson's Disease): *" Cryopreservation of iPSC - Derived Dopaminergic Neurospheres for Clinical Application* . A pioneering study demonstrating the efficacy of PROTON in dopaminergic neurospheres, with high post-thaw viability and functional recovery in a Parkinson's disease model. It confirms functional equivalence between PROTON-frozen and fresh neurospheres, including in vivo trials in rat models (improvement of motor symptoms).

2. Ayllón Rived, M. (2025) (Journal of Biomedical Advanced Science & Research): *" Scientific Validation of PROTON Magnetic Freezing Technology for Biomedical Cryopreservation* . This review/study article with multiple cases summarizes results in neurospheres (Kyoto CiRA), mesenchymal stem cells and tissues (Ryukyus), embryos, etc. It includes comparative data on viability (Table 1: PROTON 85–90% vs. LN_2 50–70 % vs. conventional 30–50%) and structural damage (Table 2: PROTON <15% damaged cells vs. up to 60% conventional). It also discusses the technical basis (citing patent EP 4063496A1) and mentions the Proton NEO project.

3. Patent JP 2025094837A (2022) – *" Method for " Producing Frozen Cells ."* Japanese patent detailing the PROTON method. The experimental appendices reported therein (and in its continuation JP 2023210612A) describe trials where increasing the field intensity improves the recovery of post-thawing spheroids, demonstrating that with PROTON at maximum intensity, the cells form spheroids and neuritic extensions almost like fresh controls, whereas with traditional methods they did not form any (total damage). These data quantitatively support the patent claims.

4. Patent EP 4 063 496 A1 (2022) – *" Cryopreservation method using electromagnetic fields for biological " Samples . "* A European patent application establishes optimized parameters for cryopreserving 3D cell aggregates, resulting from development at CiRA Kyoto. It claims the combination of cooling at ~5 °C/min with a magnetic field and RF to achieve homogeneous nucleation. This is a valuable technical resource for understanding the specific protocol applied to iPSC neurospheres.

5. Patent 2023000071 A1 (2023) – Equivalent US patent, focused on the application of the method in regenerative medicine (neurons, stem cells, organoids). It strengthens intellectual property protection in the Americas and serves as a legal reference regarding the scope of PROTON in that territory.

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Research Report

Cryopreservation of Induced Pluripotent Stem Cell-Derived Dopaminergic Neurospheres for Clinical Application

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Abstract.
Background: Pluripotent stem cell (PSC)-derived dopaminergic (DA) neurons are an expected source of cell therapy for Parkinson's disease. The transplantation of cell aggregates or neurospheres, instead of a single cell suspension has several advantages, such as keeping the 3D structure of the donor cells and ease of handling. For this PSC-based therapy to become a widely available treatment, cryopreservation of the final product is critical in the manufacturing process. However, cryopreserving cell aggregates is more complicated than cryopreserving single cell suspensions. Previous studies showed poor survival of the DA neurons after the transplantation of cryopreserved fetal ventral-mesencephalic tissues.
Objective: To achieve the cryopreservation of induced pluripotent stem cell (iPSC)-derived DA neurospheres toward clinical application.
Methods: We cryopreserved iPSC-derived DA neurospheres in various clinically applicable cryopreservation media and freezing protocols and assessed viability and neurite extension. We evaluated the population and neuronal function of cryopreserved cells by the selected method *in vitro*. We also injected the cells into 6-hydroxydopamine (6-OHDA) lesioned rats, and assessed their survival, maturation and function *in vivo.*
Results: The iPSC-derived DA neurospheres cryopreserved by Proton Freezer in the cryopreservation medium Bambanker hRM (BBK) showed favorable viability after thawing and had equivalent expression of DA-specific markers, dopamine secretion, and electrophysiological activity as fresh spheres. When transplanted into 6-OHDA-lesioned rats, the cryopreserved cells survived and differentiated into mature DA neurons, resulting in improved abnormal rotational behavior.
Conclusion: These results show that the combination of BBK and Proton Freezer is suitable for the cryopreservation of iPSC-derived DA neurospheres.

Keywords: Cryopreservation, cell-based therapy, neurosphere, dopaminergic neuron, induced pluripotent stem cells, Parkinson's disease

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INTRODUCTION

The transplantation of dopaminergic (DA) neurons is expected as a new treatment for Parkinson's disease. The proof of concept has been confirmed in previous clinical trials using fetal ventral-mesencephalic (VM) cells [1]. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are expected as alternative donor cells [2–5], and several clinical trials using these cells are ongoing (NCT024 52723, NCT03119636, JMA-IIA00384, NCT048-2733).

To make an ESC/iPSC-based therapy a widely available treatment, cryopreservation of the final product is desirable considering the logistics and manufacturing scale. Generally, the *in vivo* survival of cryopreserved cells is less than that of fresh cells. When ESC-derived DA progenitors are frozen as a single cell suspension, the survival ratio in the brain is around 60% that of fresh cells [2]. The transplantation of cell aggregates or neurospheres, instead of a single cell suspension has several advantages, such as more robust viability after the transplantation against anoikis caused by cell dissociation [6, 7], keeping the 3D structure of the donor cells and ease of handling. However, the cryopreservation of cell aggregates is more complicated than that of a single-cell suspension. Namely, it is difficult to achieve an equal distribution of the cryoprotectant (CPA) and temperature throughout the cell aggregates, increasing the risk of intracellular ice formation, which influences cell viability. Indeed, for the transplantation of cryopreserved VM tissues, the survival ratio of DA neurons is reported to be less than 20% that of fresh tissue [8–10]. Therefore, it is critical to find an effective method to cryopreserve the spheres of DA progenitors.

In general, there are two types of cell cryopreservation [11–13]. One is vitrification, an ultra-fast cooling method in which the cells are moved into liquid nitrogen immediately after treatment with a high CPA concentration [14]. It is technically challenging to apply vitrification to the manufacturing of clinical-grade cells, because it requires precise time control [15]. The other type is a slow cooling method, in which cells become frozen at about –1°C/min with a low concentration of CPA, such as 10% dimethyl sulfoxide (DMSO) [16, 17]. In this case, extracellular water freezes first, which increases the osmotic pressure of the extracellular space. The osmotic gap between the intra- and extra-cellular space causes cell dehydration. Consequently, intracellular ice formation can be reduced.

To achieve the cryopreservation of neurospheres towards clinical application, we screened various CPAs and freezing protocols from which we propose the best cryopreservation method for neurospheres to keep high viability and function.

MATERIALS AND METHODS

Maintenance and neural differentiation of human iPSCs

This study was approved by the ethical committees of Kyoto University (Kyoto, Japan) and Sumitomo Dainippon Pharma (Osaka, Japan). Human iPSC lines 1231A3 and S17 were used in the study. 1231A3 was generated under feeder-free conditions from the donor's peripheral blood using episomal vectors. S17 was established by reprogramming the donor's peripheral blood using Sendai viral vectors (CytoTune-iPS 2.0LG; ID Pharma) under GMP compliance at Sumitomo Dainippon Pharma [18]. Human iPSCs were maintained and differentiated as described in detail [19]. When we began neural differentiation, the iPSCs were dissociated into single cells after 10 min of incubation with 0.5 × TrypLE Select and were plated at 5.3×10^5 cells/cm^2 onto iMatrix511 (Nippi)-coated plates with differentiation media containing GMEM supplemented with 8% KSR, 0.1 mM MEM nonessential amino acids (all Thermo Fisher Scientific), 1 mM sodium pyruvate (Sigma-Aldrich), and 0.1 mM 2-mercaptoethanol. 0.5 × TrypLE Select was prepared by mixing TrypLE select (Thermo Fisher Scientific) and 0.5 mM EDTA/PBS. We added 100 nM LDN193189 (STEMGENT) and 500 nM A83-01 (Wako) to induce neuronal differentiation efficiently. We also added 2 μM purmorphamine (Wako) and 100 ng/mL FGF8 (Wako) from day 1 to day 7 and 3 μM CHIR99021 (Wako) from day 3 to induce floor plate cells.

Cell sorting and culture

To apply fluorescence-activated cell sorting, cultured cells were dissociated using 0.5 × TrypLE select and stained with PE-conjugated anti-CORIN antibody (100 ng/mL; Catalent/BD) for 20 min. Dead cells and debris were excluded by 7-AAD staining. The analysis was performed using a FACS Aria II or III cell sorter (BD Biosciences) and Gigasort (Cytonome). After cell sorting on culture day 12, the sorted cells were replated on low cell adhesion U bottom 96-well plates (Sumitomo Bakelite)

at a density of 3×10^4 cells per well and in neural differentiation medium containing neurobasal medium supplemented with B27 supplement, 2 mM Glutamax-I (all Thermo Fisher Scientific), 10 ng/mL GDNF, 200 μM ascorbic acid, 20 ng/mL BDNF (all Wako), 400 μM dbcAMP (Sigma-Aldrich), and 100 nM LDN193189. Unsorted cells were replated at a density of 1.5×10^4 cells per well in a neural differentiation medium without LDN193189. We changed the medium every 3 days, and 30 μM of Y-27632 (Wako) was added to the first medium. For prolonged culture, cells were cultured in the neural differentiation medium.

Cell transplantation

Animals were cared for and handled according to the Guidelines for Animal Experiments of Sumitomo Dainippon Pharma. Adult male 6-OHDA-lesioned F344 NJcl–rnu/rnu rats (CLEA Japan) were used. Cell transplantation was performed by the stereotactic injection of spheres (A,+1.0; L, –3.0; V, –5.0 and –4.0 from bregma; and TB, 0 (2 μL; 200,000 cells/μL); or A,+1.0; L, –3.5 and 2.5; V, –5.5 and –4.5 from bregma; and TB, 0 (4 μL; 200,000 cells/μL)) into the right striatum. The number of injected cells was determined by the cell count before cryopreservation. The animals were anesthetized and perfused transcardially with PBS, followed by 4% paraformaldehyde.

Behavioral analysis

The methamphetamine-induced rotation assay was performed pre-transplantation and every 4 weeks from 8 weeks after the transplantation using video-monitored rotational bowls and video tracking software (EthoVision XT; Noldus). A dose of 2.5 mg/kg of methamphetamine (Sumitomo Dainippon Pharma) was injected intraperitoneally, and the rotations were recorded for 90 min.

Cryopreservation

Spheres collected on day 28 were placed in cryovials with 1 mL ice-cold cryopreservation medium (see Table 1) and kept on ice until freezing. For the cryopreservation, the vials were transferred into a freezing container (BICELL; NIHON FREEZER), one of three programmed freezers: PDF-150 or 250 (STREX), Cryomed (Thermo Fisher Scientific), or Proton Freezer (Ryoho Freezer Systems). Six cooling profiles (shown in Fig. 3) were used. BICELL was transferred into a deep freezer (–80°C) and kept for more than 4 h. For the programmed freezers, the vials were frozen at –0.5 or –1°C/min until –40°C and then at about –(3–5)°C/min until –80°C. In the shock cooling method, the following steps were taken beginning at –4°C: freezing at –25°C/min until –35°C and then heating at +10°C/min until –12°C. Vials in Proton Freezer were kept in the chamber for 30–60 min until completely frozen. Proton Freezer combines a static magnetic field (SMF), alternating electric field (AEF), and intense airflow. After freezing, the cryovials were stored in the vapor phase of a liquid nitrogen tank. The frozen cells were quickly thawed at 37°C and diluted ten times with neurobasal medium. After supernatant removal, the cells were rinsed with PBS or saline and used for each assay or transplantation. To estimate the cell number after cryopreservation, approximately 50 aggregates were dissociated and counted by a hemocytometer to calculate the cell concentration one day after thawing and before freezing.

Quantitative RT-PCR

Total RNA was extracted using an RNeasy Mini Kit or RNeasy Micro Kit (Qiagen), and cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific). Quantitative PCR were carried out with the Fast SYBR Green PCR Master Mix in StepOne (Applied Biosystems). The data were assessed using the delta-Ct method and normalized by the *GAPDH* expression. The primer sequences used are shown in Supplementary Table 1.

Immunofluorescence studies

For the *in vitro* studies, the cultured cells were fixed with 4% paraformaldehyde. For the *in vivo* studies, fixed frozen brains were sliced at 40 μm thickness. The slices were immunologically stained using the free-floating method. The primary antibodies used are listed in Supplementary Table 2. The cells were visualized using a fluorescence microscope (BZX710 and BZX810; Keyence, AxioScan; Zeiss) and one of three confocal laser microscopes (Fluoview FV1200; Olympus, and LSM880 and LSM800; Zeiss). The number of immunoreactive cells was quantified in every 6th section throughout the grafts and corrected using the Abercrombie method. The mean intensity of the IBA1 staining was measured in the graft areas and

is shown as a ratio to the contralateral striatum. Image processing and analysis were done using Photoshop (Adobe Systems) and Fiji software.

Neurite extension assay

Floating spheres on day 28 were plated on 24-well plates coated with iMatrix511 for five days and fixed with 4% paraformaldehyde. The spheres were stained with PE-conjugated anti-PSA-NCAM antibody (1 : 100; Milteny) and visualized using the fluorescence microscope. The area covered by PSA-NCAM positive neurites was measured using Photoshop and WinRoof (Mitani Corporation).

Electrophysiological analysis

S17-derived DA neurospheres on day 28 were cultured on plates coated with poly-l-ornithine, fibronectin, and laminin (O/F/L) until whole-cell patch-clamp recordings. 1231A3-derived DA neurospheres were dissociated with papain before plating. The cells were maintained in physiological saline solution with the following composition: 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, and 17 mM glucose. Patch pipettes were made from borosilicate glass capillaries (GC150TF-10; Clark) and had a 3–4 MW resistance when filled with an internal solution composed of 140 mM KCl, 10 mM HEPES, and 0.2 mM EGTA (pH 7.3). Recordings with a voltage clamp and current clamp were made with a patch-clamp amplifier (Axopatch 200B; Molecular Devices). The giga-seal resistances were in the range of 10–20 GΩ. The current signals from the amplifier were filtered at 1 kHz and stored and analyzed on a 64-bit computer (Cooler Master). All experiments were performed at room temperature.

Multielectrode array (MEA)

Floating spheres on day 28 were cultured on laminin-coated HD-MEA chips (MaxWell Biosystems) until the recordings. The recordings were performed using the Activity Scan Assay and Network Assay modules on MaxLab Live software (MaxWell Biosystems). The entire HD-MEA was scanned using the Activity Scan Assay module, and electrodes on the spheres were selected. Spontaneous neuronal activities were recorded from the selected electrodes for 30 sec using the Network Assay module. After inactive electrodes (< 0.5 Hz) were removed, the mean values of spike amplitudes were calculated for each sphere.

Dopamine release assay

Floating spheres on day 28 were cultured on O/F/L-coated 12- or 24-well plates for 28 days, washed twice with low KCl (4.7 mM) solution, and incubated in low KCl solution for 15 min. The medium was subsequently replaced with high KCl solution (60 mM) for 15 min. The solution was then collected, and the concentration of dopamine was determined by LC-MS/MS using QTRAP6500 or Triple Quad 6500 (AB Sciex) coupled to Nexera X2 (Shimadzu). Cells that remained on the plate were harvested in PBS and sonicated. The DNA concentration of the cell lysate was measured using the Quant-iT dsDNA Assay Kit (Thermo Fisher Scientific) and used to compensate the dopamine concentration.

Statistical analysis

Statistical analyses were performed using a commercially available software package (GraphPad Prism 8; GraphPad Software). Data were analyzed by a one-way ANOVA and Tukey's *post hoc* analysis groups, an unpaired *t*-test, or a two-way ANOVA with Tukey's multiple comparisons test, as indicated in the figure legends.

RESULTS

Induction of midbrain DA progenitors from iPSCs

We induced DA progenitors from a research-grade human iPSC line (1231A3) based on a protocol with dual SMAD inhibition and floor plate induction [20]. To screen for the best cryopreservation conditions, we did not employ cell sorting to simplify the procedure (Fig. 1). The induced spheres were mainly comprised of DA progenitors (FOXA2/LMX1A; $86.1 \pm 5.1\%$) and contained some neural stem cells (SOX1; $4.7 \pm 1.6\%$ and PAX6; $2.7 \pm 0.8\%$) on day 28 (Supplementary Figure 1).

Cryopreservation with Bambanker hRM (BBK) yields good post-thaw recovery and neurite extension

First, we determined the best CPA among six clinically available ones (Table 1). After freezing

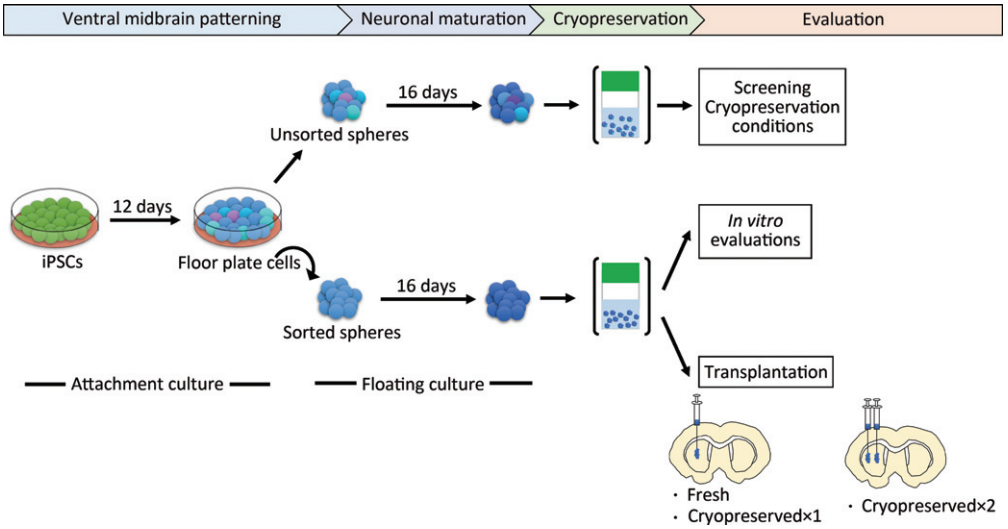

Fig. 1. Schematic overview of the protocol steps. Cryopreserved × 1 and Cryopreserved × 2 are defined in the main text. iPSCs, induced pluripotent stem cells.

the day-28 spheres at –0.5°C/min in each CPA, we thawed them and compared the cell viability and neurite extensions. We chose day 28, because we are transplanting fresh day-28 to -30 cells for Parkinson's disease patients in our clinical trial [19]. Some cells died during the freeze/thaw procedure due to apoptosis and necrosis [21], but the survived cells started to proliferate within 24 to 48 h after thawing [22–24]. Previous report indicated that measuring cell viability at 24 h post-thawing is essential to evaluate the quality and efficiency of a cryopreservation process [25]. Therefore, we examined the percentage of viable cells (viability) at 24 h, finding that BBK ($63 \pm 4\%$) gave a significantly higher percentage than STEM-CELL BANKER DMSO free (SCB DMSO-free; $21 \pm 7\%$) or CryoStor CS5 (CS5; $16 \pm 6\%$) (Fig. 2A). SCB ($49 \pm 16\%$), SaF ($49 \pm 6\%$), and CS10 ($45 \pm 14\%$) ranked second best in terms of cell recovery without any significant difference among them.

Neurite extensions are diminished by cell damage, such as neurotoxic injury and disease-related genetic aberration [26–28]. Therefore, we measured the area covered by neurites extended from the spheres five days after plating on day 28 to evaluate cell function. With BBK, the area was $49 \pm 14\%$ that of fresh spheres, a percentage significantly larger than with SCB DMSO-free or CS5 (Fig. 2B, C). Adding that BBK has already been registered in a Japanese drug master file, we concluded that BBK is the best CPA to cryopreserve DA progenitor spheres.

Proton Freezer provides the optimal freezing condition

Next, to determine the optimal freezing protocol, we froze the spheres in BBK by six different cooling conditions and two equilibration times (Fig. 3). Intracellular ice formation is a significant cause of cell death, and the control of cell dehydration and ice nucleation is a critical parameter that affects cell viability. The slower the cells are cooled, the stronger the cell dehydration and less intracellular ice. On the other hand, excessive cellular dehydration

Table 1
Commercially available xeno-free cryopreservation media tested in this study

Abbreviation	Brand name	Supplier	Major component
SCB	STEM-CELLBANKER	ZENOAQ	10% DMSO
SCB DMSO-free	STEM-CELLBANKER DMSO-free	ZENOAQ	10% Propylene glycol
BBK	Bambanker hRM	NIPPON Genetics	10% DMSO
CS5	Cryostor CS5	BioLife Solutions	5% DMSO
CS10	Cryostor CS10	BioLife Solutions	10% DMSO
SaF	Synth-a-Freeze	Thermo Fisher Scientific	10% DMSO

DMSO, dimethyl sulfoxide.

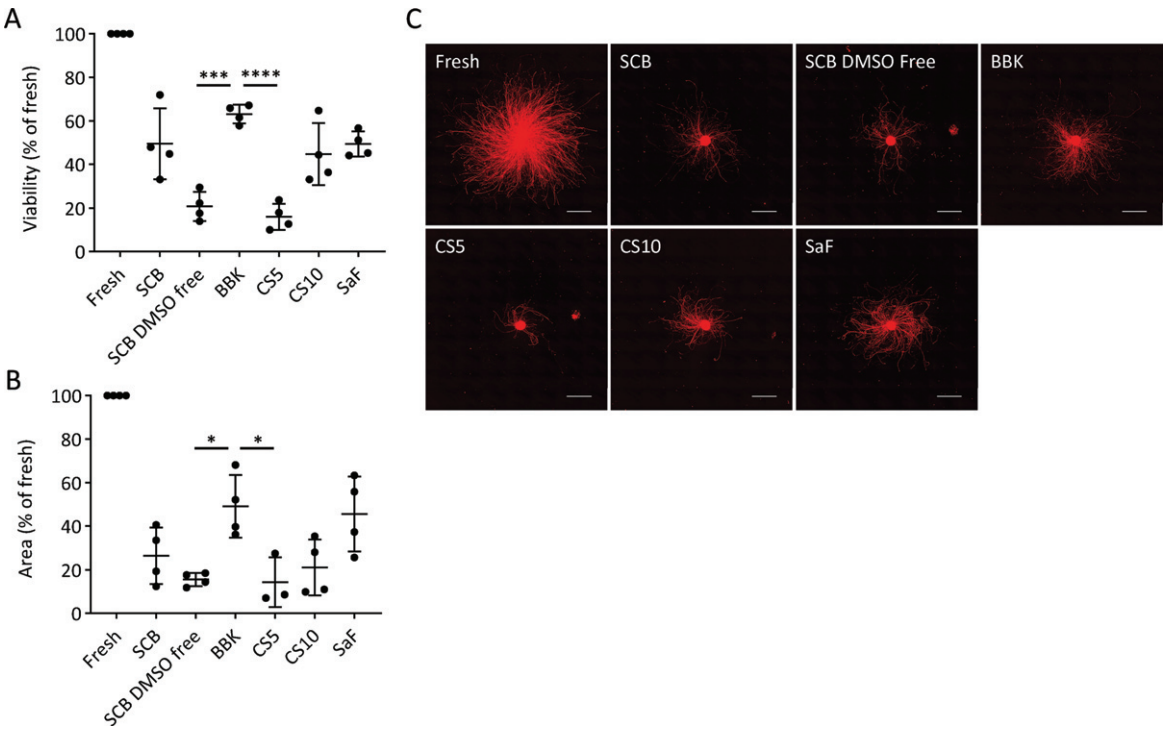

Fig. 2. Effects of cryopreservation media on iPSC-derived neurospheres (A): Viability and (B): neurite extension of spheres from unsorted cells cryopreserved at –0.5ºC/min on day 28 using the cryopreservation media shown in table 1 ($n = 4$). Viability and neurite extensions were analyzed on day 1 (24 hours) and day 5, respectively. One-way ANOVA with Tukey's multiple comparisons test; $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$ versus Bambanker hRM. C: Immunostaining of neurites for PSA-NCAM. Scale bars, 1 mm. Data are shown as means ± SD.

increases the solute concentration and damages cells [29]. Therefore, an optimal cooling rate is needed. Furthermore, during the freezing process, the CPA temperature drops below the freezing point before ice is formed (i.e., supercooling). When freezing starts, the temperature rises due to the release of latent heat and returns to the freezing point. In this process, intracellular freezing occurs because the cells freeze at once without dehydration [30, 31]. Therefore, it is essential to minimize supercooling.

BICELL is a conventional freezing container. We tested BICELL and two cooling speeds, –0.5°C/min and –1°C/min, using a programmed freezer. Next, we tried the shock cooling method. This method induces ice nucleation by a transient temperature drop and suppresses supercooling [32]. The final candidate was Proton Freezer. This freezer can rapidly cool the sample from –4°C to –30°C at –5°C/min, an intermediate speed between conventional slow cooling and vitrification. In addition, Proton Freezer includes a SMF and AEF, which align the orientation of water molecules, minimizing the ice crystals and preventing cell destruction. Finally, because the equilibration time in CPA before freezing is another critical issue to be determined, we compared 15- and 60-minute equilibration times.

We evaluated the viability and neurite extension of cells cryopreserved in BBK. At 15 minutes equilibration, there was no significant difference in viability at 24 hours between the six conditions (Fig. 4A). On the other hand, Proton Freezer showed more robust neurite extensions than the other conditions, suggesting only minor damage to the cells (Fig. 4B). At 60 min equilibration, Proton Freezer resulted in higher cell viability and neurite extensions than all other conditions (Fig. 4C, D). Neither cell viability nor neurite extensions showed a significant difference between the 15- and 60-minute equilibrations in BBK (Supplementary Figure 2).

Based on these results, we concluded that the combination of BBK and Proton Freezer is suitable for the cryopreservation of neurospheres.

Cryopreserved iPSC-derived neurospheres maintain their characteristics and functions

Next, we investigated the characteristics and functions of cryopreserved spheres derived from a

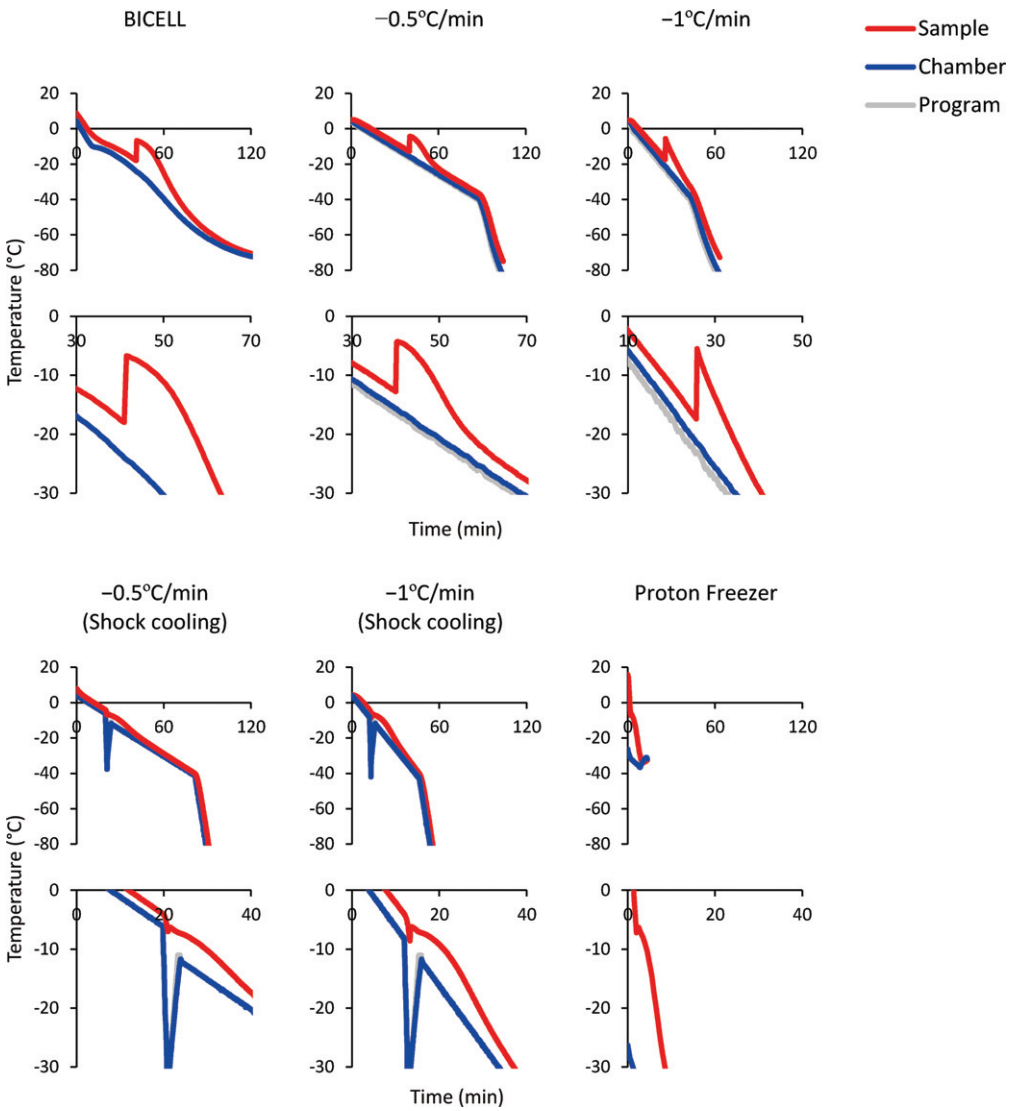

Fig. 3. Time-temperature curves of the sample (red line), freezing chamber (blue line), and program (gray line). Bambanker hRM was used as the sample. The temperature change caused by latent heat release is magnified in the lower graphs.

clinical-grade human iPSC line (S17). We sorted the cells expressing CORIN (a floor plate marker in the developing brain) on day 12 to enrich DA progenitors, cultured the sorted cells as floating spheres until day 28, and froze them in BBK using Proton Freezer (Fig. 1). We thawed the spheres and found that the cell viability and the area covered by the neurites were $63 \pm 19\%$ and $35 \pm 21\%$, respectively, compared to those of fresh spheres. To determine DA neurons, the expressions of related proteins and genes were examined seven days after thawing. Immunocytochemistry revealed that the cryopreserved spheres (D28 + 7) showed similar expressions of a floor plate marker, FOXA2, and DA neuron markers, NURR1 and TH (Fig. 5A, B), compared to fresh spheres (D35). There was no difference in the expression of two neural stem cell markers, SOX1 and PAX6, and a proliferating cell marker, KI67 (Fig. 5A, C). Quantitative PCR analyses revealed that the expression levels of several DA markers, including *FOXA2*, *LMX1A*, *EN1*, *NURR1*, *PITX3*, and *TH*, were unaffected by the cryopreservation method (Fig. 5D). In all conditions, the expression levels of the pluripotent markers *POU5F1* and *NANOG* were significantly reduced compared to undifferentiated iPSCs (Fig. 5E).

To confirm the DA function of the spheres, we conducted electrophysiological analysis and dopamine release measurements. When the cryopreserved

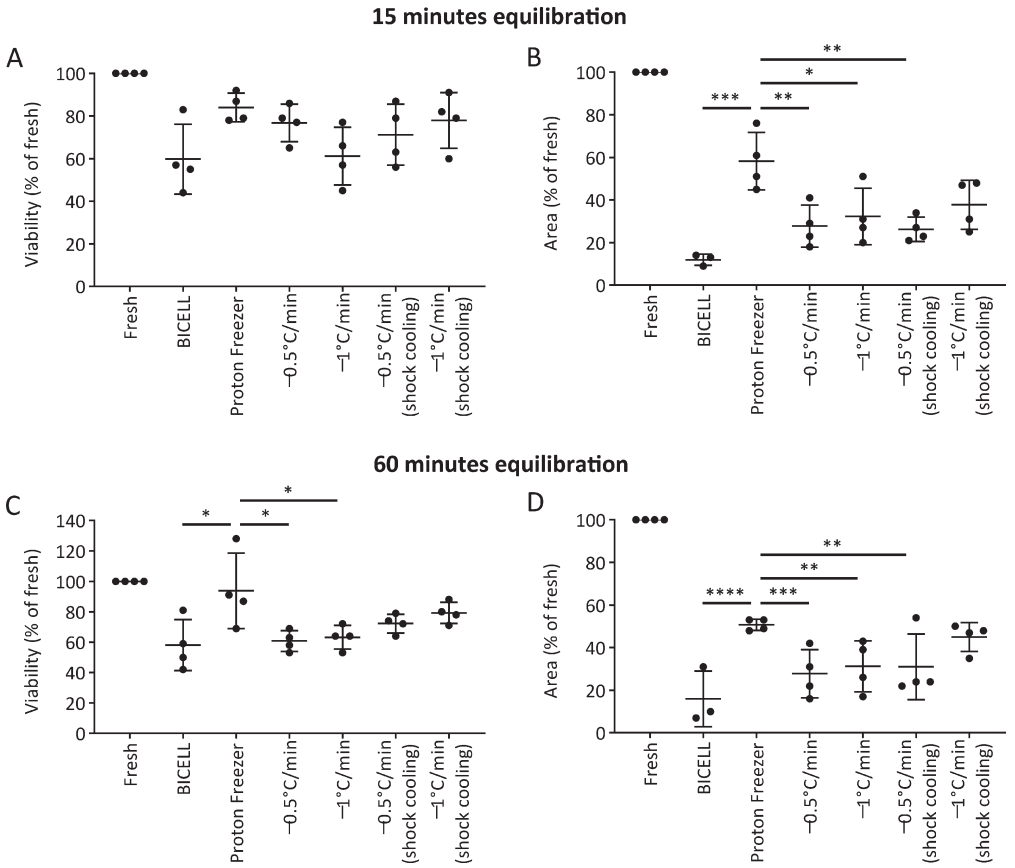

Fig. 4. Effects of the freezing program and equilibration time on iPSC-derived neurospheres. A, C) Viability and (B, D) neurite extensions of spheres from unsorted cells cryopreserved on day 28 under the different freezing programs after 15 min (A, B) and 60 min (C, D) equilibration in Bambanker hRM ($n = 4$). Viability and neurite extensions were analyzed on day 1 (24 h) and day 5, respectively. One-way ANOVA with Tukey's multiple comparisons test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ versus Proton Freezer. Data are shown as means ± SD.

spheres were cultured on the plate for further maturation, most of the cells differentiated into $TH^{+}TUBB3^{+}$ double positive DA neurons on day 49 (Fig. 5F). Repeated action potentials were detected by whole-cell current-clamp recording on day 68 (Fig. 5G). We also measured spontaneous electrophysiological activity by MEA. No difference was observed in spike amplitudes between fresh and cryopreserved cells (Fig. 5H). Dopamine secretion was measured by LC/MS/MS. The amount of dopamine released by the cryopreserved spheres on day 56 was comparable to that of the fresh spheres (Fig. 5I).

These results suggest that cryopreserved spheres can differentiate into mature and functional DA neurons as fresh spheres do. Regarding the expression of markers and functional activities, similar results were obtained using another iPSC line (1231A3) (Supplementary Figure 3).

Cryopreserved spheres improve the behavior of 6-OHDA lesioned rats

We transplanted fresh or cryopreserved spheres into 6-OHDA-lesioned rats and investigated cell survival and pharmacological efficacy. We set two dose groups for the cryopreserved spheres: cryopreserved × 1 (4×10^5 cells) and cryopreserved × 2 (8×10^5 cells). We analyzed the methamphetamine-induced rotation every four weeks. Each group with the cell transplantation showed improved abnormal behavior after 20 weeks (Fig. 6A).

An immunofluorescence study at 24 weeks revealed that the numbers of survived human cells (HNA^{+} cells) in the graft were 51,944 ± 22,462 (13.0 ± 5.6% of injected cells), 27,046 ± 8,490 (6.8 ± 2.1% of injected cells) and 63,108 ± 25,834 (7.9 ± 3.2% of injected cells) for the fresh,

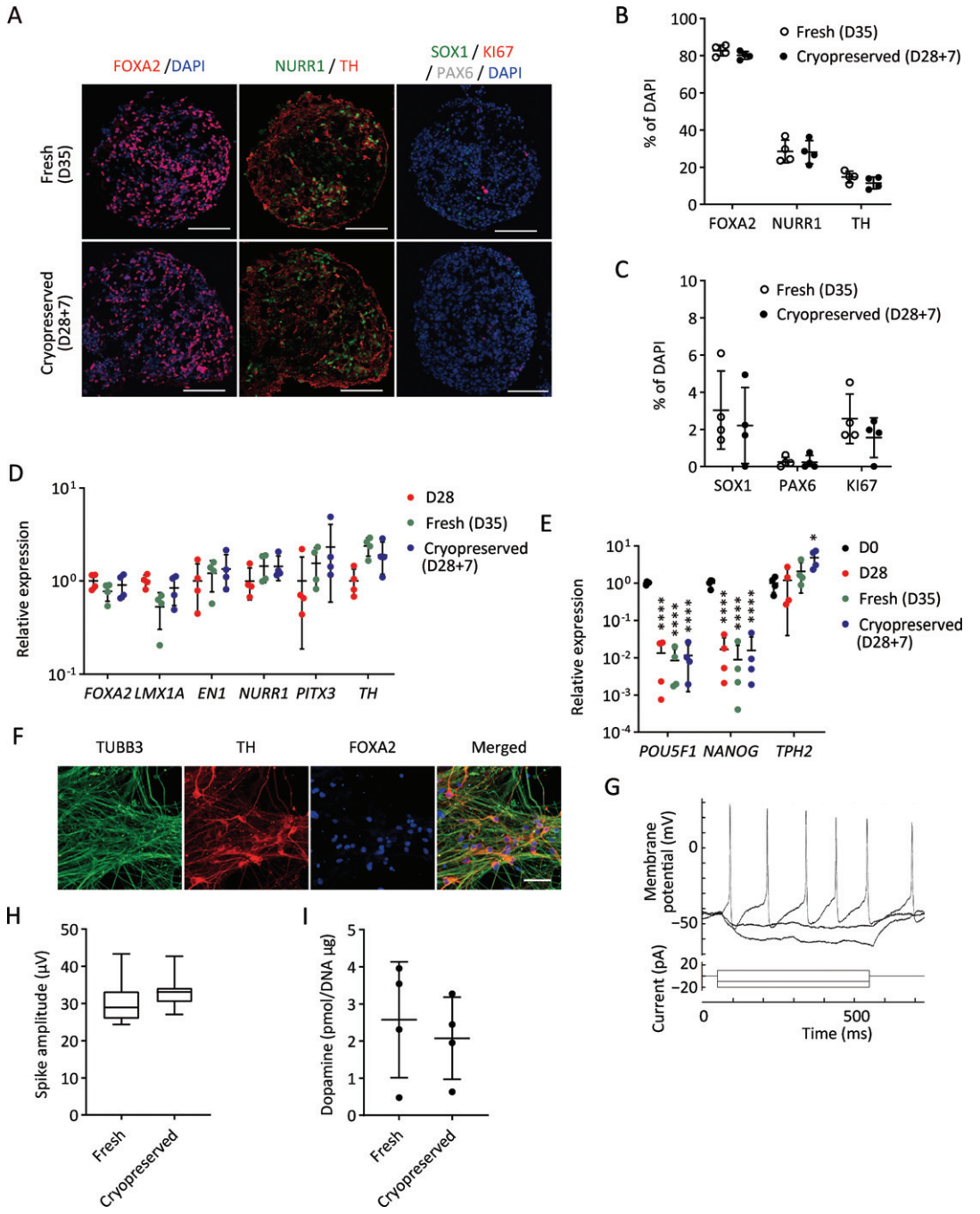

Fig. 5. Characterization of cryopreserved spheres derived from S17 *in vitro*. A) Immunostaining of the spheres on day 35. FOXA2/DAPI (left), NURR1/TH (center), and SOX1/KI67/PAX6/DAPI (right). Scale bars 100 μm. B, C) The percentages of $FOXA2^+$, $NURR1^+$, TH^+ (B) and $SOX1^+$, $PAX6^+$ and $KI67^+$ (C) cells per total cells on day 35 ($n = 4$). D, E) Gene expressions of the spheres relative to GAPDH measured by quantitative RT-PCR ($n = 4$). D28, cells cultured for 28 days; D28 + 7, cells cultured for 7 days after 28 days cryofreezing; D35, fresh cells cultured for 35 days. The expression level of D28 (D) and undifferentiated cells (D0) (E) was set to 1. There were no significant differences between D35 and D28 + 7 by one-way ANOVA with Tukey's multiple comparisons test (D). One-way ANOVA with Tukey's multiple comparisons test; $^*p < 0.05$, $^{****}p < 0.0001$ versus D0 (E). F) Immunostaining of post-thawed iPSC-derived DA neurons for TUBB3, TH, and FOXA2 on day 49. Scale bars, 50 μm. G) Representative induced action potentials of post-thawed iPSC-derived DA neurons on day 68. H) Box plots display the value in each sphere of spike amplitudes on day 42. I) The dopamine release on day 56 induced by high potassium stimulation ($n = 4$). Data are shown as means ± SD.

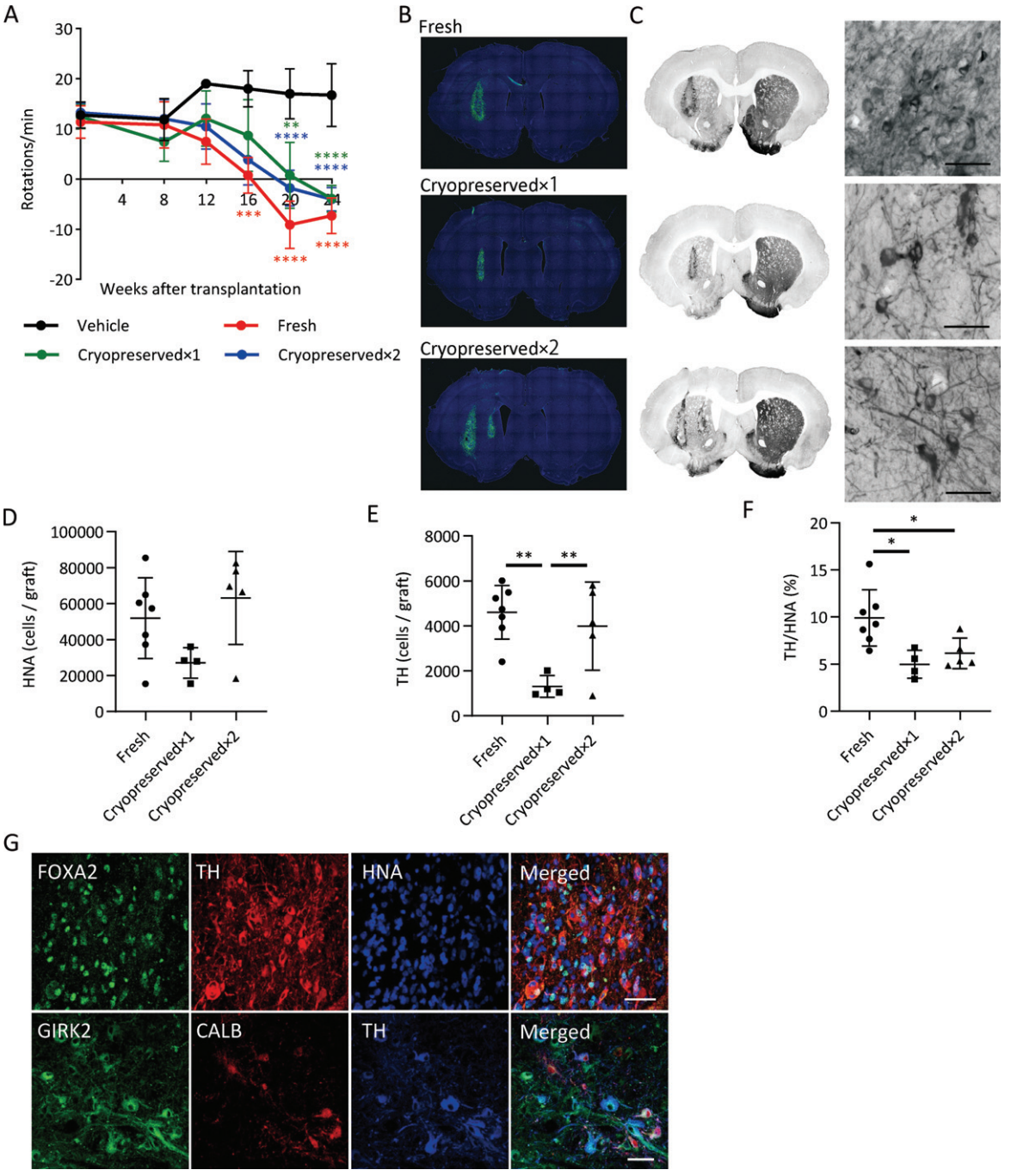

Fig. 6. Graft survival and function of cryopreserved spheres. A) The methamphetamine-induced rotations of rats that received the grafts. ($n = 4$–7). Two-way ANOVA with Tukey's multiple comparisons test; $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ versus vehicle group. B) Immunostaining of representative grafts for HNA (green) and DAPI (blue). C) DAB staining of representative grafts for TH. The right panels are magnified images of the left panels. Scale bars, 50 μm. D) The number of survived HNA^+ cells in the grafts ($n = 4$–7). E) Number of survived TH^+ cells in the grafts ($n = 4$–7). F) The percentages of TH^+ cells per survived human cells ($n = 4$–7). One-way ANOVA with Tukey's multiple comparisons test; $^{*}p < 0.05$, $^{**}p < 0.01$ (D–F). G) Immunostaining of the grafts derived from cryopreserved cells for FOXA2, TH, and HNA (upper) and GIRK2, CALB, and TH (lower). Scale bars, 50 μm. Data are shown as mean ± SD.

cryopreserved × 1 and cryopreserved × 2 groups, respectively (Fig. 6B, D), and the numbers of TH^+ cells in the graft were 4,601 ± 1,189 (1.2 ± 0.3% of injected cells), 1,306 ± 480 (0.3 ± 0.1% of injected cells) and 3,988 ± 1,961 (0.5 ± 0.2% of injected cells), respectively (Fig. 6C, E). These results suggest that the survival rate of TH^+ neurons from cryopreserved spheres is about half that compared to fresh ones. The percentages of TH^+ cells per survived human cells in the graft were relatively low in cryopreserved spheres compared to fresh ones (Fig. 6F). TH^+ cells exhibited neurite outgrowth in every group (Fig. 6C) and were positive for FOXA2 (Fig. 6G). Finally, we observed $GIRK2^+$ A9-subtype DA neurons had a large cell body with good arborization and $Calbindin (CALB)^+$ A10-subtype DA neurons had a relatively small cell body in the grafts (Fig. 6G).

To examine the immune response to the grafts, we performed staining for IBA1, a marker of microglia (Supplementary Figure 4). Activated microglia were observed around the grafts, but there was no difference in the density of microglia between the grafts of fresh and cryopreserved cells.

DISCUSSION

Here we demonstrated the advantage of combining cryopreservation medium BBK and Proton Freezer to cryopreserve iPSC-derived neurospheres for cell transplantation therapies. Several groups have reported the effects of cryopreservation on neurospheres (Supplementary Table 3). They all found good cell viability *in vitro*, but the *in vivo* survival and function in the brain are unknown [33–36]. In most cases, the spheres were cryopreserved at –(0.5–1)°C/min using DMSO as a CPA based on previous reports for the cryopreservation of neural tissues [37]. Cryopreserved VM tissues, however, often showed poor survival of DA neurons after transplantation [8–10]. Here, we established a novel, clinically applicable cryopreservation method suitable for iPSC-derived neurospheres. As shown in the previous reports about the cryopreservation of dissociated cells [2, 25, 38], the cryopreserved spheres were also equivalent to fresh spheres in the expression of DA-specific markers, dopamine secretion, and electrophysiological activity. Following their grafting into the brains of 6-OHDA lesioned rats, abnormal rotational behavior was improved.

Recently, various xeno-free, serum-free, and cGMP grade CPAs have been launched for clinical use. In the present study, iPSC-derived neurospheres cryopreserved using BBK containing 10% DMSO showed higher cell viability than other CPAs with 5% DMSO or DMSO-free (Fig. 2). For the cryopreservation of neural stem cell spheres, a higher DMSO concentration than for single-cell suspensions is suitable [39]. The reason is the difficulty in achieving uniform CPA diffusion and cell dehydration in the spheres. For most cell and tissue types, around 10% DMSO shows good cell viability [40–42], which is consistent with our results.

Next, we focused on the cooling program and revealed that Proton Freezer enabled high viability and favorable neurite extensions (Fig. 4). Proton Freezer freezes samples by a combination of weak SMF (1–200 mT), AEF in the region of radio waves (0.2–1 MHz), and cold air [43]. The application of a magnetic field (MF) and electric field (EF) to the cryopreservation has been studied and developed mainly for food preservation and used to freeze various foods, including fish and meat, and more recently biomaterials. The effects of a MF and EF on cryopreservation vary depending on the intensities, frequencies, solutions, etc., and the precise mechanisms underlying them are not entirely elucidated [44]. Previous papers suggest that SMF increases the hydrogen bonds between water molecules and makes more ordered and stable distributed water clusters, leading to the promotion of ice nucleation and suppression of supercooling [45–47]. AEF affects the vibration and orientation of water molecules and changes ice formation properties [46, 48]. The influence of AEF on ice formation is highly dependent on its frequency. When freezing in 0.9% NaCl solution, a 500 kHz frequency minimized supercooling [49], but AEF at 20 kHz or radio frequencies induced tiny ice crystals [50, 51]. Thus, Proton Freezer can minimize ice crystals by SMF and AEF. SMF aligns water molecules and prevents ice crystals from growing larger, while AEF vibrates water molecules and accelerates ice nucleation. These effects of SMF and AEF possibly improved the cell viability of iPSC-derived neurospheres.

6-OHDA lesioned rats showed complete recovery of methamphetamine-induced abnormal rotations five months after the transplantation of either cryopreserved or fresh cells (Fig. 6A). This observation suggests that cryopreserved cells in the spheres retained DA neuron function even in the brain. When comparing TH^+ cell survival per injected cell, the cryopreserved spheres showed about 50% survival compared to fresh ones (Fig. 6E). This rate is better

than previous reports, which showed less than 20% TH^+ cell survival [8–10]. Notably, the percentage of TH^+ cells per survived human cells in the graft was significantly reduced in cryopreserved spheres compared to fresh ones (Fig. 6F). Cryopreservation transiently decreases cell metabolism and biochemical reactions [24], and cryopreserved cells take a longer time for maturation after transplantation than fresh cells due to the freezing damage [52]. Considering there was no significant difference in the number of survived cells between the grafts of fresh and cryopreserved spheres (Fig. 6D), we concluded that maturation of the cryopreserved spheres was delayed.

In addition, the lower survival rate leads to the concern that cryopreserved cells may induce a more robust immune response than fresh cells because more dead cells may become present the brain. However, there was no significant difference in the immune response by the host brain (Supplementary Figure 4). This observation suggests that the possible increase in dead cells due to cryopreservation causes no significant impact on the immune response because in both cases, cryopreserved cells and fresh cells, most of the cells die after transplantation.

In conclusion, this study developed a cryopreservation method suitable for iPSC-derived DA neurospheres by introducing a unique cooling method. The cryopreserved cells maintain function as DA neurons, including pharmacological activity. Along with the freezing processes, further improvements may be possible by optimizing other parameters such as the thaw rate, cell density in the vials, freezing volume, and cryovial type (material, thickness, etc.). Importantly, our approach enables off-the-shelf availability of large-scale iPSC-derived neurospheres and can be applied to the cryopreservation of other types of tissues or cell aggregates.

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CONFLICT OF INTEREST

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SUPPLEMENTARY MATERIAL

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Scientific Validation of PROTON Magnetic Freezing Technology for Biomedical Cryopreservation

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Summary

Efficient cryopreservation of cells and tissues is a central challenge in regenerative medicine and biobanking. Conventional techniques (slow freezing at -80°C °C or in liquid nitrogen) lead to the formation of damaging ice crystals and a significant loss of cell viability (only 30–50% of cells typically survive). PROTON magnetic freezing technology, developed by Ryoho Freeze Systems Co., combines controlled cold air (35 °C) with electromagnetic fields to align water molecules and generate uniform ice nanocrystals. This minimizes osmotic and structural damage during freezing. This article validates, through a review of clinical and experimental studies, the benefits of PROTON as a superior alternative to traditional cryogenic methods. In a variety of models – including iPS cell-derived dopaminergic neurospheres for Parkinson's, fertilized oocytes, stem cells (iPS, ES, mesenchymal ADSCs), human tissues (skin, cornea), and even cryonics applications – PROTON has demonstrated post-thaw viability rates of up to 85–90%, with preservation of biological functionality and compliance with GMP /GLP standards. These results, supported by studies from leading institutions (Kyoto University, Univ. of the Ryukyus, NIBIOHN, Sumitomo Pharma), indicate that PROTON can redefine the standards of biomedical cryopreservation, offering greater efficacy and safety than cryopreservation in liquid nitrogen or at -80°C. °C.

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Cell and tissue cryopreservation is a fundamental pillar of modern medicine, but conventional methods have critical limitations. Japanese PROTON magnetic freezing technology, developed by Ryoho Freeze Systems Co., offers an innovative solution by aligning water molecules using electromagnetic fields and cold air during freezing. Unlike slow freezing at -80°C or cooling in liquid nitrogen, PROTON induces ultra-fast nucleation that forms ice nanocrystals rather than large crystals, preventing cellular

structural damage. The result is homogeneous freezing (reaching -35 °C without thermal shock) that preserves cellular integrity and minimizes the formation of harmful intracellular ice.

Numerous recent clinical and technical studies validate that PROTON outperforms conventional techniques in post-thaw cell viability, reduced cell damage, and maintenance of biological functionality. For example, iPS cell-derived dopaminergic neurospheres cryopreserved with PROTON show post-thaw viabilities greater than 85% , preserving their morphology and functional capacity to release dopamine, in contrast to typical viability of 50% or less with traditional methods pubmed.ncbi.nlm.nih.gov . Furthermore, in fertilized embryos and oocytes, PROTON achieves significantly higher survival and development rates than conventional static freezing, decreasing cell mortality and improving embryonic developmental potential. In human tissues (e.g. skin, cornea) and stem cells (mesenchymal ADSCs, pluripotent iPS/ES cells), trials (University of the Ryukyus, Japan) report viabilities of 80–90% with PROTON vs. ~40–50% with standard methods, along with lower apoptosis and maintenance of cellular functionality. Even in the field of human cryonics, PROTON emerges as a safe alternative to liquid nitrogen, achieving superior structural preservation in whole organs and tissues without the risks associated with cryogen handling. This evidence consolidates PROTON as a new standard in biomedical cryopreservation, combining biological efficacy, operational safety and compatibility with clinical environments (GMP / GLP).

The following is a scientific report that rigorously validates PROTON technology against conventional cryopreservation techniques. Comparative results are included for cell viability, structural damage, post-transplant functionality, as well as safety and regulatory compliance considerations. The data are derived from collaborations with leading institutions (Kyoto University / CiRA, University of the Ryukyus, NIBIOHN, Sumitomo Pharma) and are complemented by the scientific literature in cryobiology. The following table summarizes the observed advantages of PROTON compared to freezing in liquid nitrogen and -80°C freezers:

Criterion	PROTON	Nitrogen Liquid	Freezing - 80°C
Post-thaw viability	85–90%	50–70%	30–50%
Cellular damage (structure)	<15%	30–40%	50–60%
Use of toxic cryoprotectants	No (minimum)	Yeah	Yeah
Risk to the operator	Null	High	Half
GMP /GLP compatibility	Total	Limited	Limited
Operating cost / Logistics	Bass (electric)	High (LN_2 logistics)	Moderate (electric)
Equipment lifespan	>20 years	Variable (depends on tank)	10–12 years

Interpretation: PROTON consistently achieves higher percentages of viable cells postthaw, with less observed structural damage, compared to cryopreservation in liquid nitrogen or traditional mechanical freezers. Furthermore, PROTON does not require liquid nitrogen or high concentrations of toxic cryoprotectants, improving operational safety (eliminating risks of LN_2 burns or asphyxiation) and facilitating its integration into sterile, GMP /GLP compliant environments. From an economic perspective, PROTON systems feature low operating costs and a long shelf life, avoiding costly continuous cryogenic logistics. In summary, PROTON technology proves to be a superior alternative, poised to revolutionize biomedical cryopreservation, ensuring maximum cell viability and functionality with increased safety and efficiency.

Introduction

The ability to freeze and store living cells has enabled tremendous advances in biomedicine—from preserving cell lines and tissues for research to advanced cell therapies and germplasm banks. However, ensuring that cells survive the freezing and thawing process

intact remains a significant challenge. Traditional cryopreservation methods use slow freezing in -80°C freezers. °C or rapid cooling in liquid nitrogen (196 °C), usually in the presence of cryoprotectants such as dimethyl sulfoxide (DMSO). Both approaches have well-documented drawbacks: during freezing, intracellular water forms large ice crystals that damage cell membranes and organelles, causing dehydration and osmotic stress. As a result, post-thaw cell viability is often severely reduced (often below 50% in many protocols). This loss is especially pronounced in bulky samples or three-dimensional structures such as cell spheroids and tissues, where cryoprotectant diffusion is limited and non-uniform freezing leads to temperature gradients and internal mechanical stresses.

Classical cryobiology has recognized that minimizing intracellular ice formation is key to improving post-cryogenic survival. Strategies such as vitrification, which involves ultra-rapid cooling with high concentrations of cryoprotectants to solidify water without forming crystals, have been developed. Vitrification has been shown to significantly improve survival in certain contexts (e.g., in human embryos and oocytes, where survival rates of >85–90% after thawing have been reported) sbivf.com . However, this technique requires the use of liquid nitrogen to achieve extreme cooling rates, as well as high concentrations of cryoprotective agents (e.g., ~10–20% DMSO , ethylene glycol, sucrose), which can be cytotoxic. Furthermore, manual vitrification procedures require high skill levels and are not always easily standardized under GMP production environments.

In this context, PROTON magnetic freezing technology emerges as a disruptive proposal for biomedical cryopreservation. Unlike conventional systems, PROTON employs a static electromagnetic field superimposed on an alternating radiofrequency electric field , along with a controlled cold airflow around -35°C. °C. This unique combination of factors induces rapid and homogeneous nucleation of ice in the sample, resulting in the formation of a multitude of micro- or nanocrystals instead of a few large crystals. The alignment of polar water molecules by the magnetic field prevents chaotic ice aggregation and avoids explosive crystallization. In practical terms, PROTON freezing can achieve uniform sample cooling to approximately -35°C. °C without the thermal shocks associated with immersion in liquid nitrogen. Since large crystals do not form, mechanical damage to cells during freezing is minimized, and cell membranes and internal structures are better preserved.

PROTON technology was initially developed in Japan for food applications, proving effective in preserving the structure and quality of frozen foods (e.g., fish, meat, and delicate items like sushi) by reducing moisture loss and maintaining organoleptic characteristics. Recognizing its potential, PROTON has since been adapted for biomedical applications, obtaining certifications for use in hospital settings and biomedical research. Given the importance of improved cryopreservation methods— particularly for regenerative medicine (e.g., stem cell banking, iPS cell therapies), assisted reproduction (egg/embryo banking), organ/tissue transplantation, clinical specimen cryopreservation, and cryonics —it is crucial to benchmark PROTON's performance against conventional methods and validate its benefits.

This paper presents the results of such an assessment. Data from several key studies conducted between 2021 and 2024 applying PROTON freezing in biomedical settings were compiled and analyzed, including:

- Cryopreservation of iPSC-derived dopaminergic neurospheres for Parkinson's treatment (Kyoto University – Sumitomo Pharma CiRA collaboration) pubmed.ncbi.nlm.nih.gov;
- Human stem cell and tissue preservation (University of the Ryukyus, Okinawa) under PROTON protocols;
- A next-generation PROTON system development project (Proton NEO , in consortium with NIBIOHN and Chubu Electric Power) targeting spheroids, organoids, and clinical banks; and
- Preliminary whole-body cryopreservation studies (applied cryonics).

Additionally, PROTON's cell viability and damage metrics were compared with literature data on conventional methods. Our working hypothesis was that PROTON provides significantly higher cell viability and less post-thaw structural damage than standard techniques, while also maintaining the functionality of the cryopreserved biosystems. Below, we detail the

methodology employed and the findings obtained, followed by a discussion of their scientific relevance and practical applications.

Materials and Methods

PROTON Technology Principle: The PROTON platform consists of an electromagnetic freezer that typically operates at -30 to -35 °C. During the process, the biological sample (suspended cells, cell spheres, tissue, etc.) is simultaneously exposed to: (a) a moderate-strength static magnetic field; (b) an alternating radiofrequency electric field; and (c) a constant flow of cooled air. This combination causes an orderly alignment of water molecules (especially H_2O, whose polarity responds to the field) in the sample while reducing the temperature. Consequently, the nucleation rate is increased: numerous ice formation sites are generated almost simultaneously, producing very small and homogeneously distributed crystals. Unlike conventional freezers, in which a few initial molecules crystallize and act as seeds for the growth of large crystals, PROTON forces early massive nucleation, slowing uncontrolled crystal growth. Furthermore, precise control of heat extraction avoids steep thermal gradients in the sample, preventing mechanical stress. The expected result is a "benign" ice structure at the micro level that causes minimal damage to cells. In short, PROTON achieves a more controlled and less destructive liquid-to-solid transition than traditional methods.

Cryopreservation Protocols Evaluated

For scientific validation purposes, various freezing protocols were considered depending on the sample type, always comparing the PROTON method with a conventional reference method. In general terms, the typical steps for cryopreserving biological samples with PROTON were as follows (adapted from a protocol recently patented by CiRA /Sumitomo Pharma researchers):

- **Preparation (pre-cooling):** Cells or tissues are suspended in a compatible cryoprotective medium. For example, in the case of iPSC-derived dopaminergic neurospheres, the Bambanker hRM cryoprotectant in 10% DMSO was used, incubating the samples at +4 °C for several minutes to promote penetration of the cryoprotectant.
- **PROTON Controlled Freezing:** Samples are placed inside the PROTON unit. A gradual cooling program is applied, typically decreasing the temperature from approximately +5°C to +10°C. °C to -35 °C at a controlled rate (e.g. ~4 °C per minute, depending on the specific protocol). During this cooling, PROTON's electromagnetic fields are activated to induce uniform nucleation. In some protocols, the critical nucleation phase is controlled between -5 °C and -15 °C to ensure the formation of thin ice before reaching cryogenic temperatures.
- **Storage:** Once frozen homogeneously at around -35 °C, the samples were transferred for long-term storage either in very low temperature freezers (80 °C) or in liquid nitrogen tanks in vapor phase (above liquid LN_2, at ~-150 °C). This immediate transfer after PROTON freezing combines the benefits of controlled freezing (minimal initial damage) with the advantages of extreme low temperatures for indefinite preservation. In clinical settings, the vapor phase of nitrogen is preferred to avoid direct contact with the cryogenic liquid, reducing the risk of contamination and thermal shock.
- **Thawing:** Frozen samples were recovered by rapid thawing in a 37°C bath. °C (in vials) or at controlled room temperature, according to standard recommendations, thus minimizing recrystallization. After thawing, the cryoprotectants were gradually removed (successive dilutions) before the sample was evaluated.

Studies Analyzed

To validate PROTON's effectiveness in various biomedical fields, the results of several studies and use cases were analyzed, grouped into the following categories:

Dopaminergic neurospheres (Parkinson's)

Collaborative study between Kyoto University (CiRA) and Sumitomo Dainippon Pharma , focused on the cryopreservation of three-dimensional aggregates of dopaminergic neurons derived from human iPS cells pubmed.ncbi.nlm.nih.gov . This study included direct comparisons between conventional freezing (-80 freezer °C with DMSO) and PROTON freezing of the neurospheres, assessing cell viability, neuronal marker preservation, and dopaminergic functionality in vitro and in vivo. Information from patent

EP 4 063 496 A1 filed by these researchers, which details an optimized protocol for 3D freezing of neuronal spheres, is also considered here.

Stem Cells and Tissues (Regenerative Medicine)

Research conducted at the University of the Ryukyus (Okinawa, Japan) on the application of PROTON to cryopreserve mesenchymal stem cells (ADSCs), pluripotent stem cells (iPS and embryonic ES cells), and small human tissues (e.g., skin and cornea fragments). In these studies, post-thaw viability and histological/functional integrity were measured by comparing PROTON with conventional static freezing.

Oocytes and Embryos (Fertility)

Pilot studies on freezing fertilized eggs (early embryos) using PROTON, in collaboration with oocyte banks. Three groups were compared: embryos vitrified with PROTON, embryos frozen in a conventional -80°C freezer, and embryos frozen in a conventional -80°C freezer. °C, and a fresh, unfrozen control group. Embryo survival and blastocyst development rates were evaluated at 24, 48, and 72 hours post-thawing.

Proton NEO Project (Spheroids and Organoids):

A joint research project initiated in 2023 between NIBIOHN (National Institute of Biomedical Innovation of Japan) , Chubu Electric Power Co., and Ryoho Freeze Systems. The goal is to develop the next generation of PROTON freezers ("Proton NEO") optimized for cryopreserving complex cellular products such as organoids, large spheroids, and tissues for cell therapy. Preliminary reports of this project are reviewed, including technical improvements (e.g., ~30% reduction in freezing time compared to conventional systems through the use of electromagnetism) and initial results on organoid viability.

Human cryopreservation (Cryonics)

Although still in the exploratory stages, PROTON applications in cryonics have been considered, i.e., the preservation of whole organs and even human bodies at low temperatures for future resuscitation. There is no formal scientific literature on this subject yet, but qualitative data from internal case studies were included where PROTON was used to cool larger tissues/ organs, evaluating the reduction in macrostructural damage compared to direct immersion in liquid nitrogen.

In all of the above cases, the main evaluation criteria were: post-thaw cell viability (percentage of live cells typically assessed by trypan blue exclusion assays or similar), structural damage (e.g., membrane integrity, tissue histology, LDH release), cell/tissue functionality (e.g., ability of neurons to secrete dopamine and form synapses, embryo development potential, stem cell proliferation capacity, etc.), and safety/operability (e.g., absence of contamination, ease of handling, risks for the operator). The results obtained are presented below.

Results

Post-Thawing Cell Viability

In all models studied, PROTON technology showed a marked improvement in cell survival after thawing compared to conventional methods. Table 1 summarizes the viability percentages obtained in different systems, representing the overall findings:

Table 1: Post-Thaw Cell Viability

Freezing method	Post-thaw viability (%)
PROTON (-35 °C)	85–90%
Liquid nitrogen	50–70%
Conventional freezing (-80 °C)	30–50%

These values indicate that PROTON achieves twice or more viable cells compared to traditional static freezing, and substantially above standard cryopreservation in liquid nitrogen. For example, in the dopaminergic neurosphere study (Kyoto CiRA), the mean viability of spheres frozen with PROTON was 85–90%, while using conventional freezing (~ -80 °C with DMSO) resulted in only ~50% viable cells. Similarly, in tests with mesenchymal stem cells and tissues (Ryukyus), PROTON preserved more than 80% of live cells, compared to approximately 40% with the classical method. This substantial difference suggests that the damage induced during freezing is much lower under the PROTON regime.

Cellular and structural damage: In parallel with viability measurements, the percentage of damaged cells or cells with compromised membranes after thawing was quantified (e.g.,

propidium iodide-labeled cells, tissue histology with necrosis, etc.). The results, inversely correlated with viability, demonstrate that PROTON significantly minimizes damage.

Table 2: Presents General Estimates of Cell Damage for Each Method

Freezing method	Post-thaw structural cell damage (%)
PROTON	<15%
Liquid nitrogen	30–40%
Conventional freezing (-80 °C)	50–60%

As can be seen, PROTON-treated samples typically show less than 15% damaged cells after thawing, while with conventional methods the proportion of damaged cells can reach 30–60%. Microscopically, cells and tissues frozen with PROTON exhibited continuous membranes and intact cytoplasm, without excessive vacuolization or signs of lysis, which were common in conventionally frozen samples. In histological sections of human skin frozen with PROTON, for example, the epidermal architecture was much better preserved than in skin frozen at -80 ° C. °C, where areas of cell separation and intracellular edema were noted. These findings confirm that PROTON's controlled nucleation strategy prevents the formation of large crystals responsible for cell rupture.

Case 1 - Dopaminergic neurospheres (Parkinson)

iPSC-derived neurospheres, 200–300 µm in diameter, for Parkinson's therapy were one of the most challenging models tested. Prior to the use of PROTON, freezing these spheres in liquid nitrogen or conventional freezers resulted in poor survival and loss of dopaminergic functionality. By applying PROTON technology in conjunction with a specialized cryoprotectant (Bambanker hRM), neurosphere viability was maintained at 85–90%. More importantly, post-thaw functional testing showed that PROTON neurospheres retained their spherical morphology and expressed dopaminergic neuronal markers equivalent to never- frozen spheres pubmed.ncbi.nlm.nih.gov . In vitro dopamine release assays confirmed that they continued to secrete this neurotransmitter normally. Additionally, transplants were performed in a mouse model of Parkinson's (rats with 6- OHDA lesions): PROTON-cryopreserved neurospheres survived transplantation and integrated into the rat brain, differentiating into mature dopaminergic neurons that restored motor function in the animals (decrease in apomorphine-induced abnormal turns) pubmed.ncbi.nlm.nih.gov . In contrast, rats transplanted with neurospheres frozen by the conventional method showed poor graft survival and little behavioural improvement, corroborating the superiority of PROTON in preserving viable and functional cells. Importantly, no teratomas or uncontrolled proliferation were observed in transplants with PROTON cells, indicating that the process does not compromise the biological safety of the cells.

These results were so successful that they served as the basis for the patent application EP 4 063 496 A1, which describes in detail the 3D cryopreservation protocol developed. The patent establishes parameters such as: controlled contact of the neurospheres with the cryoprotective solution, optimal cooling rates (~5 °C/min) under a magnetic field, and criteria for preserving cell identity post-thawing. This technical breakthrough lays the groundwork for large-scale production of standardized cell therapies: thanks to PROTON, it is feasible to manufacture "batches" of dopaminergic neurospheres, cryopreserve them without significant loss of quality, and store them in banks ready for distribution to clinics, something that is not feasible with traditional cryopreservation methods. In fact, a clinical trial using cryopreserved iPSC derived dopaminergic neurons (in contrast to previous studies that used fresh cells) has already been initiated in Japan , clinically validating the strategy nature.com.

Case 2 - Stem Cells and Tissues (Ryukyus)

In the trials conducted at the University of the Ryukyus, mesenchymal stem cells (derived from human adipose tissue) and small tissue fragments (human skin and cornea) were evaluated, assessing their recovery after cryopreservation. The results reinforced the picture observed with neurospheres: with PROTON, cell survival exceeded 80%, while static methods yielded only ~40% viable cells. Similarly, apoptosis markers (active caspases, TUNEL) were significantly lower in cells thawed with PROTON, evidencing less sublethal damage. PROTON stem cells maintained their proliferative capacity: after thawing, they were able to continue dividing and forming colonies, unlike

conventionally frozen cells, which showed greatly reduced proliferation. In complex tissues such as the cornea, PROTON freezing preserved transparency and lamellar structure to a greater extent, which is promising for tissue banking or tissue engineering. These findings suggest that PROTON is particularly beneficial for sensitive and high clinical value samples, where maximizing viability is crucial (e.g., in autologous cell therapies, one often has limited amounts of cells available, so losing half during cryopreservation is very problematic).

Case 3 - Fertilized oocytes (Assisted Fertility)

Although the current standard in assisted reproduction is ultra-rapid vitrification of embryos in LN_2 , the use of PROTON as a nitrogen-free alternative was explored. In a pilot study with mammalian embryos (bovine in vitro model), embryos frozen with PROTON showed a significantly higher post-thaw survival rate than those frozen in a -80°C freezer. °C. At 24 hours post-thaw culture, more than 90% of PROTON embryos remained viable (resuming cell division), compared to <50% in the -80 group. °C (estimated study data). Furthermore, the blastocyst development rate was higher in the PROTON group, indicating that not only did more embryos survive, but they also better preserved their developmental potential. The control group of fresh embryos established the expected baseline. These preliminary results suggest that PROTON could be used to freeze embryos/oocytes with comparable efficiencies to conventional vitrification, while simplifying the process (fewer toxic cryoprotectants, no LN_2 manipulation) and increasing safety. This is attractive to fertility clinics, as PROTON would reduce staff and sample exposure to liquid nitrogen and could be standardized with automated equipment.

Case 4 - Proton NEO Project (Organoids)

Within the framework of the project with NIBIOHN and Chubu Electric, advanced prototypes of PROTON freezers called Proton NEO have been developed, targeting next-generation clinical applications. These freezers integrate PROTON electromagnetic technology with more precise control systems, allowing for specific protocols to be programmed for different sample types (e.g., liver organoids, cardiac microtissues). Initial reports indicated that Proton NEO can reduce freezing times by ~30% compared to conventional protocols, due to improvements in heat transfer and nucleation efficiency. In tests on human liver organoids (~1 mm in diameter), Proton NEO was able to freeze them uniformly without fractures and with thaw viabilities close to 80%. These industrial collaborations confirm the scalability of the technology and anticipate its early commercial availability for hospitals and biobanks (Proton NEO is reported to be ready for distribution by 2024–2025).

Case 5 - Applied Cryonics

Although still a speculative field, there are cases where cryonics institutions have shown interest in PROTON for the initial cooling phase of large bodies or organs. In one such case, PROTON was used to pre-freeze clinical-sized human liver tissue (~200 g) before transferring it to liquid nitrogen. PROTON cooling successfully lowered the tissue core to -30°C homogeneously and without visible crystallization (ice formation was monitored using Doppler ultrasound). This contrasts with traditional freezing, where large organs often crack and become ischemic before fully freezing. While vitrification is not achieved at -30°C, the formation of thin ice using PROTON could pre-condition the organ for subsequent descent to deep cryogenic temperatures with less incremental damage. In this way, PROTON could partially or completely replace the direct use of liquid nitrogen in cryonics, increasing safety (by avoiding the manipulation of LN_2 during the initial process) and reducing thermal damage. However, it is important to emphasize that the revival of cryopreserved organs or people remains hypothetical; PROTON's contribution, for now, is limited to improving the preservation process, but does not guarantee biological reversibility.

Discussion

The presented results consistently confirm that PROTON magnetic freezing technology significantly improves critical parameters of biomedical cryopreservation compared to conventional methods. First, PROTON allows much higher post-thaw cell viability rates. This is directly attributed to the reduction in physical and osmotic damage suffered by cells during the freezing phase. The controlled nucleation in PROTON prevents the formation of large ice crystals inside or outside cells, which are the main cause of membrane rupture, solute extravasation, and cell death during freezing. Our findings are in line with

other reports in the literature: for example, Mazur already postulated in 1970 that freezing sufficiently fast to avoid intracellular crystals improves survival. PROTON achieves this goal by a novel approach: instead of relying exclusively on extremely high cooling rates (as in vitrification), it uses electromagnetic fields to induce microcrystallization at moderate cooling rates. This represents an interesting hybrid approach from a cryobiology perspective: the benefits of vitrification (non-damaging ice) are obtained without requiring ultra-low temperatures or thermal shock.

Indeed, compared to conventional vitrification in assisted reproduction, PROTON offers some key operational advantages. While embryo/oocyte vitrification achieves survival rates of around 90% sbivf.com, it requires handling each sample in liquid nitrogen and exposing it to very high concentrations of cryoprotectants (e.g., ~15% DMSO + 15% ethylene glycol in the oocyte vitrification protocol), which can induce cellular toxicity and osmolarity damage. PROTON, in contrast, has been shown to achieve comparable survival rates using much lower concentrations of cryoprotectant (typically 5–10% DMSO in media like Bambanker in our studies) because the primary protection mechanism is driven by the physics of magnetic nucleation, not just the chemistry of the cryoprotectant. This reduces chemical stress on the cells. Furthermore, from the point of view of production under quality standards, PROTON integrates better into GMP environments: it is a closed, programmable equipment that can be easily validated and cleaned, while manual vitrification in straws with open LN_2 carries risks of contamination and variability between operators.

Another key aspect is the elimination of liquid nitrogen during routine processing. Liquid nitrogen has been the mainstay of cryopreservation for decades, but it carries risks: handling at -196 °C (risk of severe burns), the possibility of asphyxiation in confined spaces due to N_2 evaporation, and costly logistics (requiring cryogenic tanks, constant replenishment, etc.). PROTON operates on conventional electricity, without cryogenic gases, which increases the safety of the operator and the laboratory environment. This is particularly relevant in hospitals and clean rooms, where the presence of liquid nitrogen can be problematic. Additionally, PROTON simplifies the cold chain: since samples are optimally pre-frozen, they can be stored in mechanical freezers (ultra-freezers at -80 °C). °C) for short periods or transported in dry ice, in some cases even avoiding the use of nitrogen for shipping. For long-term storage (>1 year), vapor nitrogen tanks will likely still be used, but the volume and frequency of LN_2 handling would be drastically reduced.

Biological functionality perspective, PROTON has demonstrated something crucial: it not only keeps cells alive, but also keeps them functionally competent. In the paradigmatic case of Parkinson's neurospheres, we saw that the cells retain their specialized neuronal phenotype and can integrate into host tissues, fulfilling their function (releasing dopamine and improving motor symptoms) pubmed.ncbi.nlm.nih.gov. This suggests that PROTON also preserves cell-cell interactions in 3D aggregates , something that traditional techniques fail to achieve, as many cells die and those that survive may lose synapses or internal connections. Similarly, the ability of an embryo to resume development after thawing (form a viable blastocyst) was greater with PROTON, indicating that sublethal damage is minimized; the embryonic cells maintained their division potential. This integral preservation (viability + function) is essential for clinical applications: cells are not just allowed to survive; they must continue to serve their therapeutic purpose (whether to differentiate properly, secrete factors, contract, etc., depending on the cell type). Our data support that PROTON achieves this goal better than current alternatives.

It is worth highlighting that this technology is compatible with regulated environments (GMP /GLP) for cell production for human use. PROTON equipment can be installed in clean rooms, does not generate particles, and is easy to operate (just load the sample into the compartment, select the appropriate freezing program, and run). It does not require personnel with specialized training in cryobiology for routine operation, unlike manual vitrification, which is almost a surgical "art." This means that, with PROTON, clinical biobanks and cell therapy laboratories can better standardize their cryopreservation processes, reduce batch-to-batch variability and ensure more reliable products. Furthermore, the equipment has a long lifespan (>20

years) and requires virtually no periodic maintenance, according to the manufacturer's specifications. This results in a low amortized cost compared to the ongoing expenditure on liquid nitrogen, tank maintenance, and replacement of freezers, which typically fail in approximately 10 years.

Compared to other recent developments in cryobiology, PROTON magnetic freezing represents a unique approach. There are lines of research in isochoric cryopreservation (maintaining samples at elevated pressure to prevent ice formation) and nanoheating (to revive vitrifications with magnetic particles), among others. However, many of these approaches primarily address the thawing phase (e.g., preventing fractures during rapid rewarming of vitrified organs). PROTON attacks the problem from the outset: avoiding freeze damage, which is the initial bottleneck. In principle, PROTON could be combined with other techniques (imagine: freezing a large organ with PROTON down to -35°C, then vitrifying it with minimal amounts of cryoprotectant and perhaps rewarming it with nanoheating) to achieve currently unthinkable goals, such as ischemia-free organ cryopreservation for transplantation. At the moment, our results already confirm that in the range of cell to tissue samples (mm to cm), PROTON meets and exceeds current standards.

Some limitations should be mentioned. First, PROTON operates down to around -35°C; storage at lower temperatures (e.g., -80°C or -150°C) still requires transferring samples to ultra-low temperature freezers or nitrogen tanks after the PROTON phase. This means that the system does not completely eliminate the need for conventional cryogenic equipment for the long-term preservation phase, although it does minimize it. Second, PROTON's efficacy may depend on a complementary cryoprotectant: in our cases, some freezing medium was always used (albeit at low concentrations), so it is not proven that PROTON alone allows cryopreservation without cryoprotectants altogether. Third, more independently published research is still needed. Much of the evidence here comes from internal technical reports and patents; it would be desirable to see more peer- reviewed studies evaluating PROTON in a variety of cells (which will likely occur in the coming years given the attention the technology is gaining).

In summary, the discussion of the data leads us to conclude that PROTON brings substantial and unique advantages to the science of cryopreservation. The ability to preserve cells and tissues with high viability and functionality without the need for liquid nitrogen opens up new opportunities in treatment logistics and biobanking. For example, allogeneic (off -the -shelf) cell therapies could be produced, frozen with PROTON, and stored in centralized banks, ready to be shipped to patients on demand, with less product loss and greater safety for sample handlers. In fertility, PROTON could offer a safe alternative for small clinics without nitrogen tanks, allowing embryos to be stored locally before transferring them to a central bank. In fundamental cryobiology, PROTON provides a new physical parameter (magnetic field) to explore how to influence ice nucleation, which could inspire further innovations.

Conclusions

PROTON magnetic freezing technology represents a disruptive advance in the field of biomedical cryopreservation. Based on the evidence gathered, it is concluded that PROTON achieves:

- **Increased Post-Thaw Viability:** around 85–90% across multiple cell types, approximately doubling the rates achieved with conventional freezing. This results in much more efficient recovery of frozen biological samples, reducing critical cell loss.
- **Minimal Structural Damage:** Thanks to the formation of ice in microcrystals, cells preserve the integrity of their membranes and cytoarchitecture. Indicators of damage and apoptosis are drastically reduced in samples treated with PROTON compared to those frozen using standard methods.
- **Preservation of Biological Functionality:** Highly specialized cells (such as dopaminergic neurons) maintain their function after thawing, and tissues retain their histological viability. This is critical for clinical applications (e.g., cell therapies, tissue transplants), where not only survival but also post-cryo functional quality is important.
- **Superior Safety and Ease of Operation:** By not using liquid nitrogen or hazardous cryogels , PROTON eliminates the risk of cryogenic accidents and is friendly to the hospital environment. Your operations can be standardized to meet GMP /GLP standards without the complexity of traditional cryopreservation.

- **Logistics Chain Compatibility and Cost Savings:** Frozen samples stored with PROTON can be stored flexibly, and the equipment has a long shelf life and low maintenance requirements, resulting in lower long-term operating costs.

Together, these points position PROTON as a superior alternative to freezing in liquid nitrogen and -80 °C for most biomedical cryopreservation applications. The scientific validation presented—ranging from stem cells and embryos to neuronal aggregates and tissues—strongly supports the adoption of this technology. As more research centers and biobanks incorporate PROTON, a transformation in preservation standards can be expected: greater efficiency, greater security, and new possibilities (such as the cryopreservation of complex structures) will become reality.

At the forefront of future medicine—whether the global availability of cell therapies, fertility preservation, or even the distant prospect of cryonic organ revival —PROTON freezing is emerging as a key technological enabler. We recommend its consideration and implementation in biomedical facilities seeking to improve their cryopreservation outcomes. The data support that PROTON is not just another tool, but a paradigm shifts toward more benign, efficient, and reliable cryopreservation [1-10].

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[Document Name]Description

[Title of the invention]Method for producing frozen cells

[Technical field]

[0001]The present invention relates to a method for producing frozen cells, and the like.

[Background of the invention]

[0002]In recent years, research and development of cell pharmaceuticals using iPS cells have been advanced. For example, the use of spheroids of dopamine neurons for the treatment of Parkinson's disease has been reported (Non-Patent Documents 1 and 2). This technology has already started a physician-driven clinical trial. However, currently used cells are cells that have not undergone a freezing step from differentiation induction to spheroid formation and neurite outgrowth. When cells that have not been subjected to such a freezing step are used, it is difficult to store the cells for a long period of time after completion of culture, and therefore, consistent schedule management from production of the cells to administration of the cells to a patient is essential.

[Prior art documents]

[Non-patent document]

[0003]

[Non-patent document 1]Takahashi J, Regenerative Therapy 13,18-22, 2020

[Non-patent document 2]Journal of Parkinson's Disease 12 (2022) 871 - 884

[Summary of the invention]

[Problem to be solved by the invention]

[0004]An object of the present invention is to provide a cell freezing technique capable of maintaining cell functions (for example, spheroid-forming ability and neurite outgrowth ability after freezing and thawing) in a higher state.

[Means for solving the problem]

[0005]As a result of intensive studies in view of the above problems, the present inventors have found that the above problems can be solved by a method for producing frozen cells, the method including freezing cells under radiation of radio waves having an electric field intensity of 100V/m or more. That is, the present invention includes the following aspects.

[0006]Item 1. A method for producing frozen cells, comprising freezing cells under radiation of radio waves having an electric field intensity of 100V/m or more.

[0007]Item 2. The production method according to Item 1, wherein the cell is a neural cell.

[0008]Item 3. The production method according to Item 2, wherein the neural cell is at least one selected from the group consisting of a neural stem cell, a neural progenitor cell, and a nerve cell.

[0009]Item 4. The method according to Item 2 or 3, wherein the neural cell is derived from a stem cell.

[0010]Item 5. The production method according to Item 4, wherein the stem cells are induced pluripotent stem cells.

[0011]Item 6. The method according to any one of Items 2 to 5, wherein the neural cell comprises a single cell or a cell mass.

[0012]Item 7. The method according to any one of Items 1 to 6, wherein the electric field intensity of the radio wave is 150V/m or more and 300V/m or less.

[0013]Item 8. The production method according to any one of Items 1 to 7, wherein the cells are frozen at -20 ° C or lower.

[0014]Item 9. The production method according to any one of Items 1 to 8, wherein the cells are frozen at -100 ° C or higher and - 20 ° C or lower.

[0015]Item 10. The production method according to any one of Items 1 to 9, wherein the cells are frozen in the presence of a magnetic field.

[0016]Item 11. The method according to Item 10, wherein the magnetic field has a magnetic field strength of 5 μ T or more.

[0017]Item 12. The method according to Item 10 or 11, wherein the magnetic field has a magnetic field strength of 5 μ T or more and 50 μ T or less.

[0018]Item 13. A frozen cell obtained by the production method according to any one of Items 1 to 12, or a thawed cell of the frozen cell.

[0019]Item 14. A medicament comprising thawed cells of frozen cells obtained by the production method according to any one of Items 1 to 12, or differentiation-induced cells of the thawed cells.

[Effect of the invention]

[0020]According to the present invention, it is possible to provide a cell freezing technique capable of maintaining a cell function (for example, spheroid-forming ability, neurite outgrowth ability) in a higher state, a cell obtained by the technique, a medicament containing the cell, and the like.

[Brief description of the drawings]

[0021]

FIG. 1 shows the results of Test Example 1. The vertical axis indicates the average value of the sphere size. The horizontal axis indicates the cell type used. The

legend indicates the freezing conditions.

FIG. 2 shows the results of Test Example 2. Cell types are shown on the left side of the photograph. The device / instrument used for freezing is shown at the top of the photograph.

FIG. 3 shows a photographic image of the results of Test Example 3. The device / instrument used for freezing is shown at the top of the photograph.

FIG. 4 shows the results of measurement of the area of the neurite portion in Test Example 3 (vertical axis). On the horizontal axis the device / instrument used for freezing is indicated. * * * * indicates that the P value for comparison between groups is less than 0.0001.

[Mode for carrying out the invention]

[0022]In the present specification, the expressions "contain (s)" and "comprise (s)" include the concepts of "contain (s)", "comprise (s)", "consist (s) substantially of", and "consist (s) only of".

[0023]In the present specification, when an upper limit and a lower limit are indicated, not only a range defined by the upper limit and a range defined by the lower limit, but also a range obtained by arbitrarily combining the upper limit and the lower limit can be adopted.

[0024]1. Method for Producing Frozen Cells In one aspect, the present invention relates to a method for producing frozen cells, comprising freezing cells under radiation of radio waves having an electric field intensity of 100V/m or more (sometimes referred to as "the production method of the present invention" in the present specification). This will be described below.

[0025]The cells to be frozen in the production method of the present invention are not particularly limited. According to the production method of the present invention, cells can be frozen while maintaining higher cell functions (e.g., spheroid-forming ability and neurite outgrowth ability of cells after thawing). Examples of the cells include blood cells, hematopoietic stem cells / hematopoietic progenitor cells, gametes (sperms, eggs), fibroblasts, epithelial cells, vascular endothelial cells, nerve cells, liver cells, keratinocytes, muscle cells, epidermal cells, endocrine cells, pluripotent stem cells (ES cells, iPS cells, etc.), tissue stem cells, cancer cells, and the like.

[0026]The cells to be frozen in the production method of the present invention are particularly preferably nervous system cells. Neural cells are particularly expected to be used for cell transplantation because they do not regenerate in vivo. Therefore, there is a particular need for a method of cryopreserving cells for transplantation prepared in vitro while retaining their functions. According to the production method of the present invention, cells can be frozen while maintaining the cell function (for example, spheroid-forming ability, neurite outgrowth ability) of neural cells in a higher state.

[0027]The neural cells include neurons (Neuronal cell or Neuron), precursor cells of the neurons, that is, neural precursor cells (neural progenitor cell or neural precursor cell), neural stem cells, and the like.

[0028]The neural cell may be a neural cell derived from any site such as a neural cell of the central nervous system, a somatic neural cell of the motor nerve or sensory organ system, or a neural cell of the peripheral nervous system such as a neural cell of the autonomic nerve system, and examples thereof include a nerve (neuron), a neural crest-derived cell, a glial cell such as an oligodendrocyte or an astrocyte, and a stem cell or a precursor cell thereof. Neural cells include cells that express neural cell markers. Examples of the marker for neural cells include, but are not limited to, NCAM, β III-Tubulin (TUJ1), tyrosine hydroxylase (TH), serotonin, nestin, MAP2,MAP2AB,NEUN,GABA , glutamate, CHAT,SOX1,BF1,EMX1,VGLUT1,PAX,NKX,GSH,Telencephalin,GLUR1,CAMKII,CTIP2,TBR1,Reelin,TBR1,BRN2,OTX2,LMX1A,LMX1B,EN1,NURR1,PITX3,DAT , and GIRK2. Neural cells can be identified by expression of one or more neural cell markers. As used herein, neural cells include cells that express one or more, two or more, or three or more of the above neural cell markers.

[0029]Neural cells of the central nervous system can be classified according to the difference in the site where the neural cells are present. That is, examples thereof include neurons derived from the forebrain, telencephalon, diencephalon, cerebrum, hypothalamus, midbrain, hindbrain, midbrain-hindbrain boundary region, cerebellum, retina, pituitary, or spinal cord, and precursor cells thereof.

[0030]Forebrain derived neurons are neurons present in the forebrain tissue (i.e., telencephalon, cerebrum, hippocampus or choroid, diencephalon, hypothalamus, etc.). Neurons of the forebrain can be identified by the expression of forebrain neuron markers. Examples of the forebrain neuron marker include OTX1 (forebrain), BF1 (also referred to as FOXG1), SIX3 (also a marker of telencephalon or cerebrum), and the like. As used herein, neural cells include cells that express one or more, two or more, or three or more of the aforementioned forebrain neuron markers, telencephalon or cerebrum markers.

[0031]Examples of the cerebral nerve cell include dorsal cells (e.g., cerebral cortex cells, Cajal-Retzius cells, hippocampal nerve cells, etc.) or ventral cells (e.g., basal ganglia cells, etc.). Examples of the ventral cerebral neuron marker include basal ganglia neuron markers (e.g., GSH2,MASH1,NKX2.1,NOZ1). Examples of the dorsal cerebral neuron marker include a cerebral cortical neuron marker (e.g., PAX6,EMX1,TBR1). In the present specification, examples of the neural cell include a cell expressing one or more, two or more, or three or more of the above-described cerebral nerve cell marker, basal ganglia nerve cell marker, or cerebral cortex nerve cell marker.

[0032]Examples of the midbrain-derived neural cells include ventral midbrain-derived neural precursor cells, dopamine-producing neural cells (also referred to as dopamine neural cells or Dopaminergic neuron), dopamine-producing neural precursor cells (also referred to as dopamine neural precursor cells or Dopaminergic progenitor), and the like. Examples of the marker for mesencephalic-derived neural cells include FOXA2,EN2,TUJ1 . Examples of FOXA2 - and TUJ1 - positive neural cells include dopamine-producing neural precursor cells and dopamine-producing neural cells. In addition, dopamine-producing neurons can be identified using FOXA2 positivity, NURR1 positivity, and TH positivity as indicators.

[0033]In addition, the dopamine-producing neural progenitor cells can be identified using FOXA2 positivity and LMX1A positivity as indicators. More preferably, it contains cells that are positive for one or more of OTX2,LMX1A,LMX1B,CORIN,SHH,AADC,βIII - Tubulin,EN1,NURR1,PITX3,DAT,GIRK2 and TH. In

the present specification, the dopamine-producing neural progenitor cell may include a dopamine-producing nerve cell, a dopaminergic neuron, or the like, unless otherwise specified.

[0034]Examples of the neurons derived from the midbrain-hindbrain boundary region include neurons present in the cerebellum, cerebellar plate tissue, ventricular zone, rhombic lip, and the like. Examples of the midbrain-hindbrain boundary region marker include EN2 (midbrain), GBX2 (hindbrain), and N-Cadherin (neural progenitor cells of midbrain-hindbrain boundary region). Examples of cerebellar neural progenitor markers include GABAergic neural progenitor markers such as KIRREL2,PTF1A and SOX2, and cerebellar granule progenitor markers such as ATOH1 and BARHL1. In the present specification, examples of the neural cell include a cell expressing one or more, two or more, or three or more of the midbrain-hindbrain boundary region marker, the cerebellar neural progenitor cell marker, the GABAergic neural progenitor cell marker, or the cerebellar granule cell progenitor cell marker.

[0035]Examples of the neural cells derived from the retina include photoreceptor cells and photoreceptor precursor cells.

[0036]Neural cells can also be classified according to the difference in the neurotransmitter produced (secreted), and examples thereof include dopamine-producing nerve cells, dopamine-producing nerve precursor cells, GABA nerve cells, GABA nerve precursor cells, choline nerve cells, choline nerve precursor cells, serotonin nerve cells, serotonin nerve precursor cells, glutamate nerve cells, glutamate nerve precursor cells, noradrenaline nerve cells, noradrenaline nerve precursor cells, adrenaline nerve cells, adrenaline nerve precursor cells, and the like.

[0037]Examples of the neural cells of motor nerves and sensory organs include choline nerve cells or precursor cells thereof.

[0038]Examples of the neural cell of the autonomic nerve include a choline nerve cell, an adrenaline nerve cell, and precursor cells thereof.

[0039]Preferred examples of the neural cell herein include a dopamine-producing nerve cell (dopamine nerve cell) and a dopamine-producing nerve precursor cell (dopamine nerve precursor cell).

[0040]The neural cells derived from a living body are cells isolated from a mammal such as a human, and examples of cells isolated from human brain tissues include cells contained in middle brain tissues of a fetus as described in Nature Neuroscience,2,1137(1999) or N. Engl. J. Med.; 344:710 9 (2001).

[0041]The neural cell is also preferably a cell obtained by inducing differentiation from a stem cell, particularly preferably from a pluripotent stem cell such as an embryonic stem cell (ES cell) and an iPS cell, and differentiation induction can be appropriately performed by a method known to those skilled in the art. Methods for differentiating and inducing neural cells from stem cells include, for example, the method described in WO2015/034012 (dopamine-producing neural progenitor cells), WO2009/148170 (cerebral and other neural cells), WO2013/065763,WO2016/013669 or WO2017/126551 (pituitary or hypothalamic neural cells), WO2016/039317 (cerebellar neural cells), WO2015/076388 (telencephalic neural cells), Numasawa - Kuroiwa, Y et al., - 661 (2014) (neural progenitor cells), Qiu, L et al., - 1814 (2017) (dopamine-producing neural progenitor cells), tep (dopamine-producing neural progenitor cells), tep et al., - tep (dopamine-producing neural progenitor cells),

tep et al., Stem Cell Reports, 2: 648 Stem Cells. 31(8): 1548 Nat Biotechnol. 37(4):436 Stem Cells Transl Med. 6(9): 1803 Nolblant, S Kuwahara, A 2019 Cederquist, Y 1979 (2017 WO2013/067362 Nat Protoc. 12(9):1962 1562 (2013) Sundberg, M Sci Rep. 12;9(1):18936 (2019) (visual cells Photoreceptor precursor cells) ,WO2018/074567 (peripheral neurons).

[0042]The pluripotent stem cell is a stem cell having pluripotency capable of differentiating into many cells present in a living body and also having proliferation ability, and includes any cell induced into an intermediate mesodermal cell used in the present invention. Examples of pluripotent stem cells include, but are not particularly limited to, embryonic stem (ES) cells (human embryonic stem cells are preferably established from human embryos within 14 days of fertilization), embryonic stem (ntES) cells derived from cloned embryos obtained by nuclear transfer, spermatogonial stem cells (GS cells), embryonic germ cells (EG cells), induced pluripotent stem (iPS) cells, cultured fibroblasts, and pluripotent cells derived from bone marrow stem cells (Muse cells). Preferred pluripotent stem cells are iPS cells, more preferably human iPS cells, from the viewpoint that the effect of maintaining the cell function by the production method of the present invention is particularly remarkable, and from the viewpoint that the pluripotent stem cells can be obtained without destroying embryos, eggs, and the like in the production process.

[0043]Methods for producing iPS cells are known in the art, and iPS cells can be produced by introducing a reprogramming factor into any somatic cell. Here, examples of the reprogramming factors include genes or gene products such as Oct3/4,Sox2,Sox1,Sox3,Sox15,Sox17,Klf4,Klf2,c - Myc, N-Myc, L - Myc,Nanog,Lin28,Fbx15,ERas,ECAT15 - 2,Tcl1,beta - catenin,Lin28b,Sall1,Sall4,Esrrb,Nr5a2,Tbx3 , and Glis1, and these reprogramming factors may be used alone or in combination. Examples of the combination of reprogramming factors include WO2007/069666,WO2008/118820,WO2009/007852,WO2009/032194,WO2009/058413,WO2009/057831,WO2009/075119,WO2009/079007,WO2009/091659,WO2009/101084,WO2009/101407,WO2009/102983,WO2009/114949,WO2009/117439,WO2009/126250,WO2009/126251,WO2009/126655,WO2009/157593,WO2010/009015,WO2010/033906,WO2010/033920,WO2010/042800,WO2010/050626,WO2010/056831,WO2010/068955,WO2010/098419,WO2010/102267,WO2010/111409,WO2010/111422,WO2010/115050,WO2010/124290,WO2010/147395,WO2010/147612,Huangfu D,et al . (2008), Nat.Biotechnol.,26:795 - 797,Shi Y,et al . (2008), Cell Stem Cell,2:525 - 528,Eminli S,et al . (2008), Stem Cells.26:2467 -. (2008), bain -. (2008), tbu - tbu. (2008), pabu - tbu. (2008), pabu - tbu. (2009), tbu - tbu. (2009), tabu - tbu. (2009), tabu - tbu. (2009), tabu - tbu. (2009), tabu - tbu. (2010), Nat.Biotechnol.,27:459 8917,Kim JB,et al Cell Stem Cell,3,568 Nat.Biotechnol.26:1269 643,Ichida JK,et al 1275,Shi Y,et al 461,Lyssiotis CA,et al Proc Natl Acad Sci U S A.106:8912 Cell Stem Cell.5:491 2474,Huangfu D,et al 203,R.L.Judson et al Cell Stem Cell,3,132 Cell Stem Cell,3:475 574,Zhao Y,et al Nat.Cell Biol.11:197 Nature.461:649 479,Marson A 135,Feng B,et al 503,HengJC,et al Cell Stem Cell.6:167 - 74,Han J,et al . (2010), Nature.463:1096 - 100,Mali P,et al . (2010), Stem Cells.28:713 - 720,Maekawa M,et al . (2011) Examples thereof include the combinations described in Nature.474:225 9.

[0044]The cell (particularly, a neural cell) may be a single cell or a cell mass (including a two dimensional sheet-like structure) in which two or more cells are adhered or aggregated. When a single cell is included, it is suitable to subject the cells of the single cell to spheroid formation after freezing and thawing. In the case of containing a cell mass (particularly, a spheroid), after freezing and thawing, the cell mass is suitable for differentiation into a more highly differentiated state such as neurite outgrowth in vitro or in vivo (for example, in a

living body after cell transplantation).

[0045]A single cell is literally a cell that exists in a state of not adhering to other cells. In one aspect of the present specification, examples of the cells to be frozen include a cell population of a single cell, but in the cell population, a cell mass in which a small number of cells (for example, 5 or less, specifically 2, 3, 4, or 5 cells) are aggregated may be partially present. A single cell can be obtained by treating cells cultured in a culture vessel with a drug such as trypsin as necessary and stirring the cells by pipetting or the like.

[0046]The number of cells constituting the cellular aggregate is not particularly limited, but is usually 500-150000 , and in one embodiment, for example, 1000-100000 , 1000-70000 , or 3000-30000 .

[0047]Spheroids refer to cellular aggregates (Cell aggregate or spheres), which are three dimensional populations of cells formed by cultured cells adhering to each other. Spheroids also include self-organized so-called organoids Cellular aggregates of neural cells are also referred to as neurospheres (Neurosphere). The shape of the spheroid is not particularly limited, and may be spherical or non-spherical. The spheroid in the present specification preferably has a three dimensional shape close to a spherical shape. The three dimensional shape close to a spherical shape is a shape having a three dimensional structure, and exhibits, for example, a circular shape or an elliptical shape when projected on a two dimensional plane.

[0048]The size of the spheroids is not particularly limited, but is usually the circle-equivalent 150μm-1000μm , and in one embodiment, for example, the 200μm-800μm or the 300μm-500μm . Spheroids usually contain 500-150000 cells, for example, 1000-100000 cells, 1000-70000 cells, or 3000-30000 cells in one embodiment.

[0049]The production method of the present invention includes freezing cells under radiation of radio waves having an electric field intensity of 100V/m or more.

[0050]Typically, freezing is carried out by placing a container (vial for cryopreservation) containing a cryopreservation solution containing cells under cell freezing conditions.

[0051]In the present application, the cryopreservation solution (preservation solution) means an aqueous liquid containing a cryoprotectant. Cryoprotectants refer to substances that have a high affinity for water molecules and are highly effective in inhibiting the growth of ice crystals in a cryopreservation solution, and include, for example, dimethyl sulfoxide (DMSO), ethylene glycol (EG), propylene glycol (PG), 1,2-propanediol (1, 2-PD), 1,3-propanediol (1, 3-PD), butylenes glycol (BG), isoprene glycol (IPG), dipropylene glycol (DPG), and / or glycerin. In the present application, the cryoprotectant is preferably dimethyl sulfoxide and / or propylene glycol. The concentration of cryoprotectant in the cryopreservation solution is usually 7-12%, preferably about 10%, when dimethyl sulfoxide and / or propylene glycol are used as cryoprotectants.

[0052]Examples of the aqueous liquid include buffer solutions such as saline and PBS,EBSS,HBSS , culture solutions for culturing cells, tissues, and the like such as DMEM,GMEM,RPMI , serums, serum-substitutes, mixtures thereof, and the like.

[0053]As the cryopreservation solution, a commercially available cryopreservation solution containing dimethyl sulfoxide (DMSO) and / or propylene glycol as a

substantial component can be used. Specific examples of the cryopreservation solution include commercially available cryopreservation solutions such as STEM - CELL BANKER (SCB;ZENOAQ),STEM - CELL BANKER DMSO free (SCB DMSO free;ZENOAQ),Bambanker hRM (BBK;NIPPON Genetics),CryoStor CS5 (CS5;BioLife Solutions),CryoStor CS10 (CS10;BioLife Solutions) and Synth-a - Freeze (SaF;Thermo Fisher Scientific). For example, it is desirable to use a cryopreservation solution containing 7-12%, preferably about 10%, dimethyl sulfoxide and / or propylene glycol (e.g., STEM - CELL BANKER,Bambanker hRM,CryoStor CS10 , Synth-a-Freeze, and the like). More preferably, Bambanker hRM can be used.

[0054]As used herein, when cells are frozen, the number of cells relative to the cryopreservation solution (the cell density) is the 80000-5000000cells/mL,100000-4000000cells/mL or the 200000-2000000cells/mL,300000-1000000cells/mL .

[0055]As used herein, the volume of cells and preservation solution is 0.2mL-2mL,0.5ml-1.5ml , or from 0. 5ml to 1ml.

[0056]In the present specification, the cells and the preservation solution may be filled in a 0.5ml-15ml,1ml-5ml or a vessel from 1ml to 2ml.

[0057]The material of the container is not particularly limited as long as it is not a material that completely shields radio waves, but a material having excellent radio wave permeability is desirable. Examples of the material of the container include plastics such as polypropylene.

[0058]The freezing point of the cryopreservation solution in the present application is not particularly limited, but is usually - 1 ° C. to - 10 ° C., preferably - 3 ° C. to - 10 ° C., more preferably - 3 ° C. to - 6 ° C., and even more preferably about - 5 ° C.

[0059]As the cryopreservation solution in the present specification, those known to those skilled in the art can be appropriately used, and a commercially available cryopreservation solution can also be appropriately used. Specifically, for example, an aqueous liquid containing 7 to 12%, preferably about 10% of dimethyl sulfoxide as a substantial component and having a freezing point of - 1 ° C. to - 10 ° C. may be mentioned. Examples of the cryopreservation solution in the present specification include an aqueous liquid containing 7 to 12%, preferably about 10% of dimethyl sulfoxide as a substantial component and having a freezing point of - 3 ° C. to - 6 ° C.

[0060]The cell-freezing conditions in the production method of the present invention include being irradiated with radio waves having an electric field intensity of 100V/m or more (Condition 1).

[0061]The electric field intensity is an electric field intensity measured in the vicinity of a place where the cryopreservation vial is disposed. The electric field intensity is preferably 120V/m or more, more preferably 150V/m or more, further preferably 170V/m or more, still further preferably 180V/m or more, particularly further preferably 190V/m or more, particularly still further preferably 195V/m or more, particularly preferably / m or more, from the viewpoint that the cellular function (e.g., spheroid-forming ability, neurite-elongating ability) can be maintained in a higher state. 200VThe upper limit of the electric field intensity is not particularly limited, but is, for example, 500V/m,400V/m,300V/m or 250V/m. The

electric field intensity is, for example, 120 V/m or more and 500V/m or less, preferably 150 V/m or more and 500V/m or less, more preferably 170 V/m or more and 500V/m or less, further preferably 180 V/m or more and 500V/m or less, still further preferably 190 V/m or more and 400V/m or less, especially further preferably 195 V/m or more and 300V/m or less, and particularly preferably 200 V/m or more and / m or less. 250V

[0062]A method of measuring the electric field intensity is, for example, as follows. A plurality of (3 to 5) electromagnetic wave-measuring sensors are placed at the positions where the vials are to be placed, and measurements are taken at ambient temperature (25 ° C.) using a measuring instrument (Gaussmeter RIGOL DSA815 or equivalent), and the mean value of the measured values at the plurality of positions is calculated.

[0063]As the radio wave, for example, a radio wave included in a radio wave region having a frequency of about 300kHz to about 2MHz, preferably about 500kHz to about 1MKHz, and more preferably about 600kHz to about 1MKHz is preferable.

[0064]The radio wave irradiation method is not particularly limited, and a method in accordance with or based on a known method can be adopted. The radio wave is usually irradiated using a radio wave transmitting antenna. As the radio wave transmitting antenna, various conventionally known antennas can be used, but it is preferable to use a coil antenna from the viewpoint that a radio wave can be more reliably transmitted even with low power and the size does not become too large in consideration of the frequency described later. In addition, the coil antenna can be formed of an electric wire, a coaxial cable, or the like, and various electric wires and coaxial cables can be used from the viewpoint of insulation, dielectric constant, cold-resistant temperature, and dimensions. Examples of the electric wire include an electric wire composed of a copper wire and an external insulating coating (for example, made of fluororesin) covering the copper wire. Examples of the coaxial cable include a coaxial cable composed of an inner conductor made of copper, an insulator made of polyethylene covering the inner conductor, an outer conductor composed of a braided conductor made of copper covering the insulator, and an outer insulating film (for example, made of fluororesin) covering the outer conductor. The number of turns, thickness, and the like of the coil antenna can be appropriately selected by those skilled in the art in consideration of the frequency.

[0065]In the production method of the present invention, cells are frozen at a temperature equal to or lower than the freezing point of the cryopreservation solution. From this viewpoint, the cell freezing conditions in the production method of the present invention preferably include freezing the cells at - 20 ° C. or lower (condition 2).

[0066]The freezing temperature is preferably - 25 ° C. or lower, more preferably - 30 ° C. or lower. The lower limit of the temperature is preferably -100 ° C., more preferably - 85 ° C., still more preferably - 60 ° C., yet still more preferably - 45 ° C., and particularly preferably - 40 ° C.

[0067]The cell freezing conditions in the production method of the present invention preferably include freezing the cells in the presence of a magnetic field (condition 3).

[0068]The magnetic field strength is the magnetic field strength measured in the vicinity of the location where the cryopreservation vial is placed. The magnetic

field strength of the magnetic field is not particularly limited, but is, for example, 5 μ T or more, preferably 10 μ T or more, and more preferably 15 μ T or more. The upper limit of the magnetic field strength is not particularly limited, and is, for example, 50μT,40μT,30μT or 25 μ T. The combination of the upper limit and the lower limit of the magnetic field strength is arbitrary, but the magnetic field strength is, for example, 5 μ T or more and 50 μ T or less, preferably 5 μ T or more and 40 μ T or less, more preferably 10 μ T or more and 40 μ T or less, further preferably 10 μ T or more and 30 μ T or less, still further preferably 15 μ T or more and 30 μ T or less, and particularly preferably 15 μ T or more and 25 μ T or less.

[0069]A method of measuring the magnetic field strength is, for example, as follows. A plurality of (3 to 5) magnetic flux density measurement sensors are disposed at the positions where the vials are disposed, measurement is performed at room temperature (25 ? C.) using a measuring instrument (magnetic flux density measuring device), and the average value of the measured values at the plurality of positions is calculated.

[0070]The method for applying a magnetic field is not particularly limited, and a method according to or based on a known method can be adopted. The magnetic field is preferably a unidirectional and substantially uniform static magnetic field. The "unidirectional and substantially uniform static magnetic field" means that the lines of magnetic force run substantially linearly in one direction and the magnetic flux density is substantially constant. The magnetic field can be applied by placing a magnet body around the vial. The magnet body is not particularly limited as long as it can generate a static magnetic field having the above-described magnetic field strength, and various magnets can be used. As a magnet that can be used for the magnet body, it is preferable to use a permanent magnet such as an alnico magnet, a ferrite magnet, or a neodymium magnet from the viewpoint of continuously maintaining the properties as a magnet for a relatively long period of time without receiving a magnetic field or a current from the outside. Among them, a ferrite magnet, which is inexpensive because iron oxide is used as a main raw material, has high magnetic permeability, and is used for various applications, is preferable. The alnico magnet is a magnet (cast magnet) obtained by casting aluminum (Al), nickel (Ni), cobalt (Co), or the like as a raw material, and iron, copper, or the like may be added as an additive. A ferrite magnet is obtained by adding a small amount of barium, strontium, or the like to iron oxide as a main raw material, sintering the iron oxide, pulverizing the sintered iron oxide, molding and sintering the pulverized iron oxide, and magnetizing the sintered iron oxide with an electromagnet. The neodymium magnet is a rare-earth magnet containing neodymium, iron, and boron as main components. Among these, ferrite magnets, which are inexpensive due to the use of iron oxide as a main raw material, have high magnetic permeability, and are used in various applications, are preferable.

[0071]The time for freezing the cells under the above conditions is not particularly limited as long as the cells are frozen, and is, for example, 10 minutes to 5 hours, preferably 15 minutes to 3 hours, and more preferably 20 minutes to 2 hours.

[0072]After the cells are frozen under the above-described conditions, the cells can be stored as they are under the conditions, but are preferably transferred to and stored under conditions under which the cells are not thawed (for example, conditions of − 80 ° C. or lower, preferably − 150 ° C. or lower, such as liquid nitrogen or a freezer).

[0073]The frozen cells can be appropriately thawed and used. The thawing method is not particularly limited, but it is desirable to thaw at a temperature of about body temperature for a short time from the viewpoint of function / activity and cell survival rate. Specifically, it is desirable to thaw at a temperature of 30 ° C. to 40 ° C., preferably 35 ° C. to 38 ° C., and more preferably a temperature near the human body temperature, for example, about 37 ° C.

[0074]In one aspect, the present invention also encompasses a frozen cell obtained by the production method of the present invention, or a thawed cell of the frozen cell.

[0075]2. Medicament In one aspect, the present invention relates to a medicament comprising a thawed cell of a frozen cell obtained by the production method of the present invention, or a differentiation-induced cell of the thawed cell (sometimes referred to as "medicament of the present invention" in the present specification). This will be described below.

[0076]Differentiation induction of thawed cells can be carried out according to the method described in "1. Method for producing frozen cells" above.

[0077]When the cell is a neural cell, examples of the target disease include spinal cord injury, motor neuron disease, multiple sclerosis, amyotrophic lateral sclerosis, Huntington's chorea, multiple system atrophy, spinocerebellar degeneration, Alzheimer's disease, retinitis pigmentosa, age-related macular degeneration, and parkinsonism (including Parkinson's disease).

[0078]The medicament of the present invention contains a therapeutically effective amount of the above-mentioned cells. For example, 1 ? 104 to 1 ? 108 cells are contained for a single administration. The medicament of the present invention may contain a component such as dimethyl sulfoxide (DMSO) or serum albumin for the purpose of protecting cells, or a component such as an antibiotic for the purpose of preventing contamination with bacteria.

[0079]The route of administration of the medicament of the present invention is not particularly limited. For example, administration is by intravenous, intraarterial, intraportal, intradermal, subcutaneous, intramuscular, or intraperitoneal injection. Instead of systemic administration, local administration may be adopted. Examples of the local administration include direct injection into a target tissue, organ, or organ. The administration schedule may be prepared in consideration of the sex, age, body weight, pathology, and the like of the subject (patient). In addition to a single dose, a plurality of doses may be administered continuously or periodically.

[Examples]

[0080]Hereinafter, the present invention will be described in detail based on Examples, but the present invention is not limited to these Examples.

[0081]Test Example 1. Evaluation of spheroid-forming ability after freezing treatment 1 Dopamine neuron differentiation-induced cells differentiated from stem cells were subjected to freezing treatment by various methods, and the spheroid-forming ability after freezing treatment was examined. Specifically, the measurement was performed as follows.

[0082]Stem cells (Ff-I01s04 cells (human iPS cells, established by Center for iPS Cell Research and Application, Kyoto University, available from Kyoto University iPS

Cell Research Foundation: https://www.cira - foundation.or.jp/j/research - institution/ips - stock - project/homozygous.html), 1231A3 cells (human iPS cells:), and KhES-1 cells (human ES cells:)) were seeded in a single-cell state in a 24-well plate (trade name: Nunc 24-well plate, round / flat-bottomed, manufactured by Nunc, coated with iMatrix-epa manufactured by Matrisome Corporation), and cultured for 13 days in a differentiation-inducing medium (medium composition: evag-evag). 1x106 cells https://cellbank.brc.riken.jp/cell_bank/CellInfo/?cellNo=HPS0381&lang=En 5.3x105cells/cm2 ThermoFisher ME, 8% Knockout SerumReplacement https://cellbank.brc.riken.jp/cell_bank/CellInfo/?cellNo=HES0001&lang=Ja Glasgow MEM,NEAA, Pyruvate, 2 511 silk 0.5 μg/cm2 On day 0 from the start of culture, Y27632 was added to the medium (final concentrations: 20 μ M). On day 0 to day 6 from the start of culture, a- 83 01 was added to the medium (final concentration: 500 nM). On day 0 to day 12 from the start of culture, LDN193189 was added to the medium (final concentrations: 100 nM). On day 1 to day 6 from the start of culture, Purmorphamine, and FGF8 were added to the medium (final concentrations: 2μM, 100ng/mL). On day 3 to day 12 from the start of culture, CHIR99021 was added to the medium (final concentrations: 3 μ M). During the culture, the medium was exchanged every day. After completion of the culture for 13 days, the cells were dissociated into single cells using trypsin, the medium was removed, the cells were suspended in a cryopreservation solution (Bang-Banker (registered trade name) hRM, manufactured by Nippon Genetics Co., Ltd.), and the suspended solution was dispensed (3 - 8 x105 cells / 200 μL / vial) into vials for frozen storage (trade name: manufactured by Nalgene Cryoware Cryogenic Vial,Thermo scientific). The vial for cryopreservation was set in a proton freezer (manufactured by Chikubo Freeze Systems Co., Ltd.) modified so as to be irradiated with radio waves having a high electric field strength, and the cells in the vial were frozen. The specifications of the proton freezer are as follows.
-100V 15A
-Approximately 90cm (front-width) ? approximately 60cm (depth) x90cm (height)
-Weight: about 80kg (cold storage + proton system)
-Fixed (magnetic field strength near the vial: 18 μ T)
-Electric field intensity of radio wave: variation (0 to 10 scale)
-Three stages (strong, medium, weak)
-Temperature: -20 ° C. to -45 ° C.

[0083]A method of measuring the electric field intensity is as follows. A plurality of (3 to 5) electromagnetic wave-measuring sensors were placed at the positions where the vials were to be placed, and measurements were taken at ambient temperature (25 ° C.) using a measuring instrument (Gaussmeter RIGOL DSA815), and the mean value of the measured values at the plurality of positions was calculated.

[0084]The method of measuring the magnetic field strength is as follows. A plurality of (3 to 5) magnetic flux density measuring sensors were disposed at the positions where the vials were disposed, measurement was performed at room temperature (25 ? C.) using a measuring instrument (magnetic flux density measuring device), and the average value of the measured values at the plurality of positions was calculated.

[0085]The freezing conditions were the three patterns shown in Table 1.

[0086]

[Table 1]

	電波の電界強度 （バイアル周辺部）	庫内温度	冷風の風速
Proton 10	202.72V/m	-35℃	6～8m/s
Proton 5	67.99V/m	-35℃	6～8m/s
Proton 0	1.12V/m	-35℃	6～8m/s

[0087]The vials were frozen in a freezer for 1 hour and then plunged into liquid nitrogen for complete freezing (1 day). The vial was then placed in a 37 ° C. water bath to thaw the cells. The obtained cells were seeded in a 96-well U-bottom low-attachment plate (trade name: PrimeSurface 96 U plate, manufactured by Sumitomo Bakelite Co., Ltd.) (cells contained in 1 vial were suspended in 1300uL and dispensed to each 150μL/ well), and cultured in a spheroid-forming medium (medium composition: Neurobasal, B27, 200μM L - glutamine, 200μM Ascorbic acid, 400μM dbc AMP, 10 ng/mL GDNF, 20 ng/mL BDNF, 20μM Y27632) for 4 days. After completion of the culture, 7 or 8 wells were observed for samples of the same cell type and the same freezing conditions, and spheres were recognized with Incucyte (registered trademark) (manufactured by Sartorius Japan K. K.). The area of the sphere having the largest area in each well was defined as the sphere size of the well.

[0088]The results are shown in FIG. 1. As shown in FIG. 1, it was found that the sphere size of the spheroid formed after thawing is improved when the electric field intensity of the radio wave irradiated during freezing is increased. The sphere size in the case of Proton 10 was about the same as that in the case where spheroids were formed in the same manner using cells before freezing. From this, it was found that it is possible to perform freezing while maintaining the cell function in a higher state by increasing the electric field intensity of the radio wave irradiated during freezing.

[0089]Test Example 2. Evaluation of spheroid-forming ability after freezing treatment 2 As stem cells, 201B7 Ff cells (human iPS cells (feeder-free): https://cellbank.brc.riken.jp/cell_bank/CellInfo/?cellNo=HPS0063&lang=Ja) and 1231A3 cells (human iPS cells) were subjected to dopaminergic neural differentiation-inducing culture in the same manner as in Test Example 1, and the cells were dispensed into vials for frozen storage. The vial for cryopreservation was set in the proton freezer used in Test Example 1 (the freezing conditions were Proton 10 in Table 1), a small program freezer (PF-NP - 200, manufactured by Nepa Gene Co., Ltd.), or a cell freezing container (CoolCell (registered trademark), manufactured by Corning Incorporated), and the cells in the vial were frozen. The freezing temperatures of the small program freezer and the cell freezing container were both - 80 ° C., and the cooling rate of the small program freezer was - 1 ° C. / min.

[0090]After the freezing was completed, it was poured into liquid nitrogen for complete freezing (8 days). The vial was then placed in a 37 ° C. water bath to thaw the cells. The obtained cells were seeded in a 96-well U-bottom low-attachment plate (trade name: PrimeSurface 96 U plate, manufactured by Sumitomo Bakelite Co., Ltd.) (cells contained in 1 vial were suspended in 1300uL and dispensed to each 150μL/ well), and cultured in a spheroid-forming medium (medium composition: Neurobasal, B27, 200μM L - glutamine, 200μM Ascorbic acid, 400μM dbc AMP, 10 ng/mL GDNF, 20 ng/mL BDNF, 20μM Y27632) for 4 days. After completion of the culture, a plurality of wells were observed for samples of the same cell type under the same freezing conditions.

[0091]FIG. 2 shows an observed image of a well showing typical features of each sample. Spheroid formation could be confirmed only in the case of the proton freezer (freezing conditions were Proton 10 in Table 1), but spheroid formation could not be confirmed in the case of using other devices and instruments.

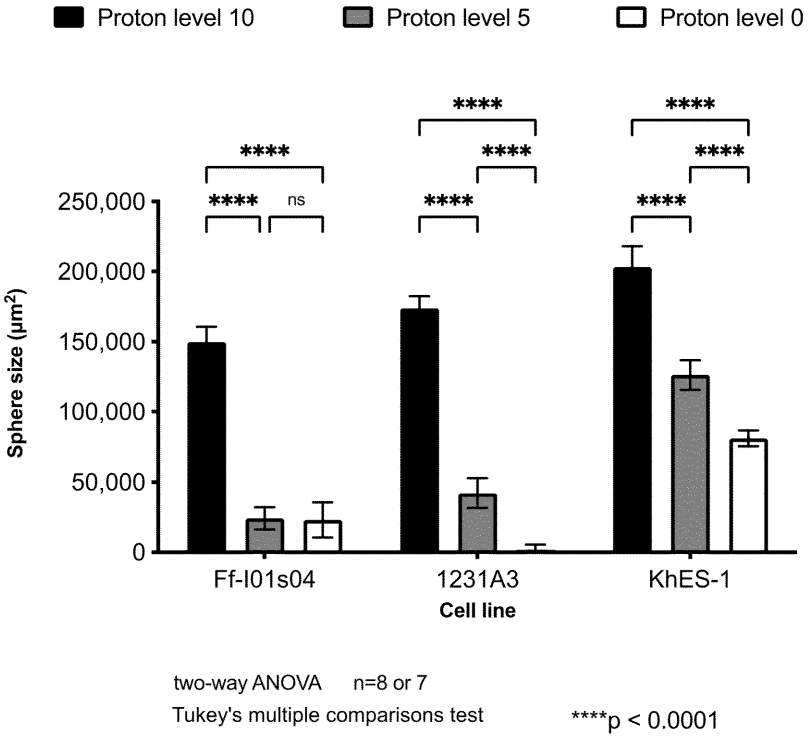
[0092]Test Example 3. Evaluation of Neurite Outgrowth Ability after Freezing Treatment Dopamine neuron differentiation-inducing culture was performed in the same manner as in Test Example 1 using 1231A3 cells (human iPS cells) as stem cells. After completion of the culture for 13 days, the cells were dissociated into single cells using trypsin, then seeded in a 96-well U-bottom low-attachment plate (trade name: PrimeSurface 96 U plate, manufactured by Sumitomo Bakelite Co., Ltd.) (cells contained in 1 vial were suspended in 1300uL and dispensed into each 150μL/ well), and cultured in a spheroid-forming medium (medium composition: Neurobasal, B27, 200μM L / glutamine, 200μM Ascorbic acid, 400μM dbc AMP, 10 ng/mL GDNF, 20 ng/mL BDNF) for 14 days. For the first three days of the culture, Y27632 was added to the medium to a final concentration of 20 μ M. The formed spheroids were dispensed into vials for frozen storage (trade name: Cryotube Inner Cap 1. 8mL non-self-standing type, round bottom type, manufactured by Nunc) in a cryopreservation solution (Bang-Banker (registered trade mark) hRM, manufactured by Nippon Genetics Co., Ltd.). The vial for cryopreservation was set in the proton freezer used in Test Example 1 (the freezing conditions were Proton 10 in Table 1), a small program freezer (PF-NP - 200, manufactured by Nepa Gene Co., Ltd.), or a cell freezing container (CoolCell (registered trademark), manufactured by Corning Incorporated), and the cells in the vial were frozen. The freezing temperatures of the small program freezer and the cell freezing container were both - 80 ° C., and the cooling rate of the small program freezer was - 1 ° C. / min.

[0093]After the freezing was completed, it was poured into liquid nitrogen for complete freezing (8 days). The vials were then thawed by mixing with PBS warmed to 37 ° C. The spheroids were seeded as they were in a 24-well plate (trade name: Tissue Culture Plate VTC - P24, manufactured by Bioramo Inc.) and subjected to adherent culture in a medium (medium composition: Neurobasal, B27, 200μM L - glutamine, 200μM Ascorbic acid, 400μM dbc AMP,10 ng/mL GDNF, 20 ng/mL BDNF, 20μM Y27632) for 3 days. After completion of the culture, the spheroids were immunostained with primary antibodies (trade name: Purified anti - Tubulin β -, manufactured by 3 (TUBB3)Antibody,BioLegend) and secondary antibodies (trade name: Donkey anti - Rabbit IgG(H+L) Highly Cross - Absorbed Secondary Antibody,Alexa Fluor (registered trade mark), manufactured by 488,Invitrogen) to evaluate the neurite outgrowth. The area of the neurite portion was measured by image analysis.

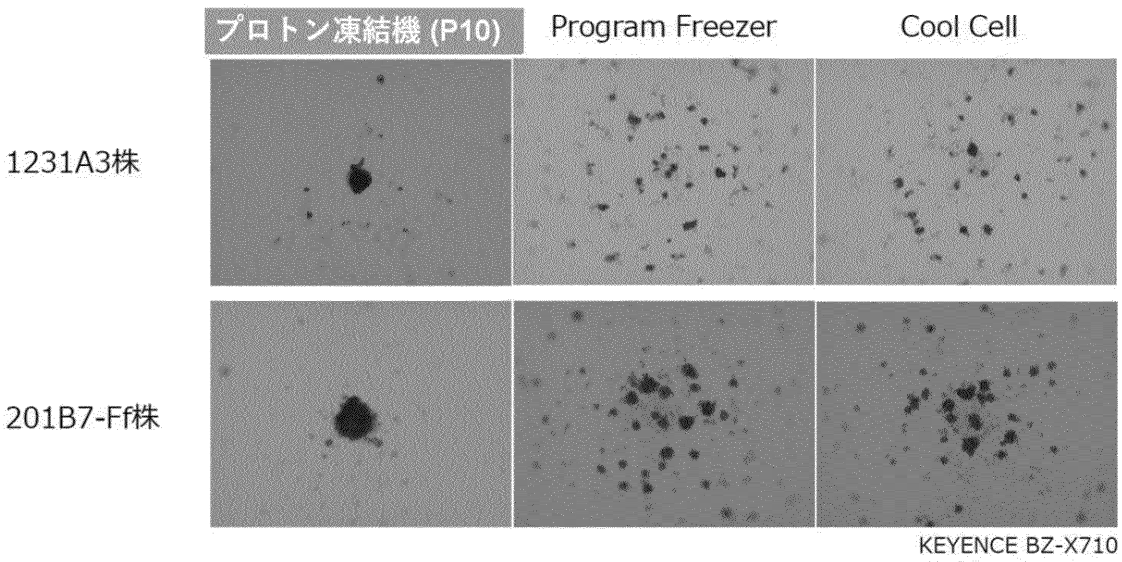
[0094]The results are shown in FIGS. 3 and 4. Neurite outgrowth was greatest with the proton freezer (freezing conditions were Proton 10 in Table 1).

[Document Name]Drawings

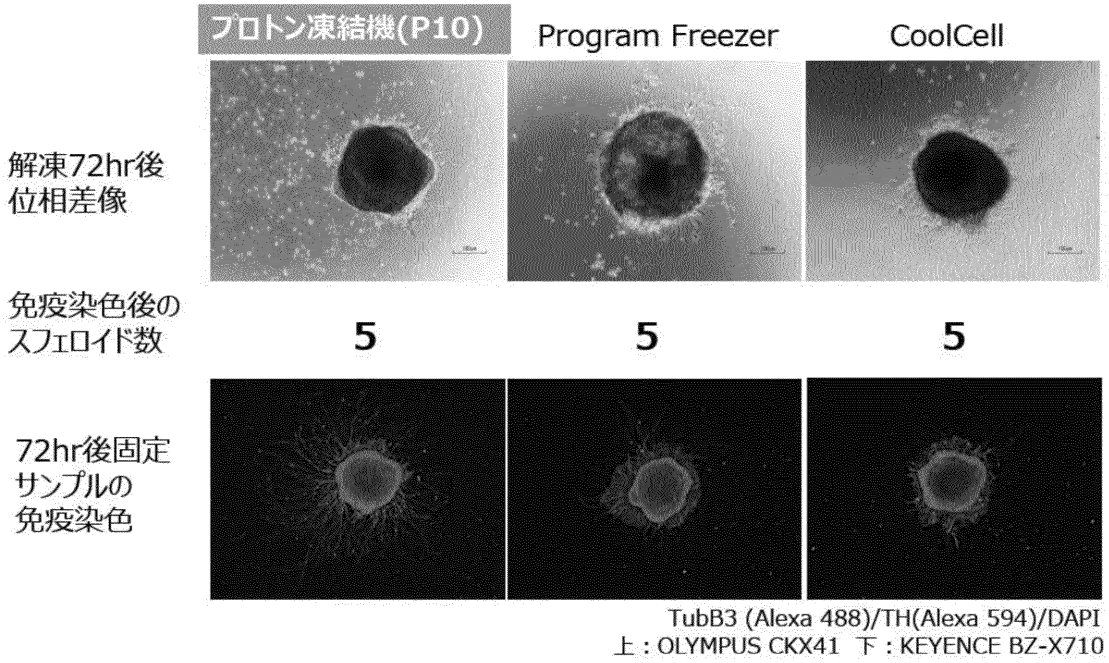
[Fig. 1]



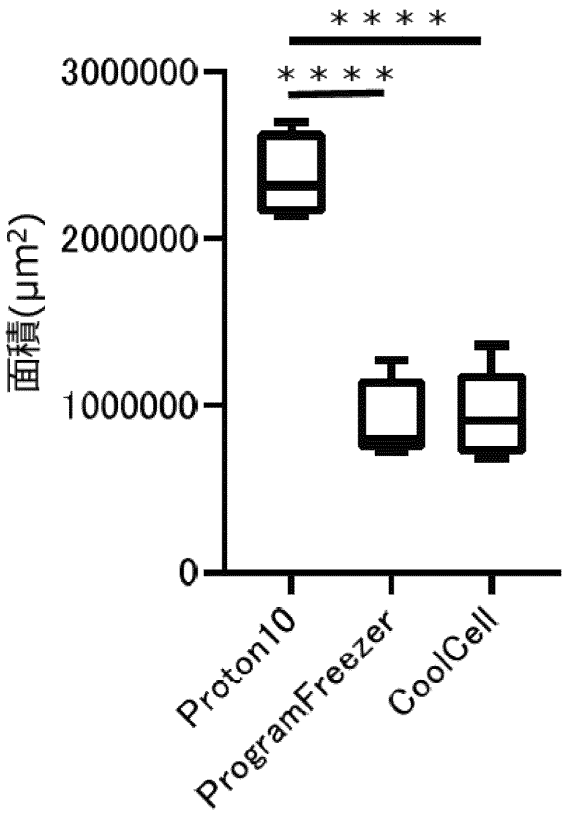
[Fig. 2]



[Fig. 3]


プロトン凍結機(P10)
Program Freezer
CoolCell
解凍72hr後
位相差像
免疫染色後の
スフェロイド数
5
5
5
72hr後固定
サンプルの
免疫染色
TubB3 (Alexa 488)/TH(Alexa 594)/DAPI
上：OLYMPUS CKX41　下：KEYENCE BZ-X710

[Fig. 4]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3000000
2000000
1000000
0
面積(μm²)
Proton10
ProgramFreezer
CoolCell

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(54) METHOD FOR FREEZING NEURAL CELLS

(57) Provided is a method for freezing a cell aggregate including neural cells. provided is a method for freezing a cell aggregate including neural cells and having a three-dimensional structure, which comprises following steps (1) and (2): (1) contacting a cell aggregate including neural cells and having a three-dimensional structure with a preservation solution at 0°C to 30°C prior to freezing to prepare a preservation solution-soaked cell aggregate; and (2) cooling the preservation solution-soaked cell aggregate obtained in step (1) from a temperature at least about 5°C higher than the freezing point of the preservation solution to a temperature about 5°C lower than the freezing point at an average cooling speed of 2 to 7 °C/min to freeze the cell aggregate.

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Description

TECHNICAL FIELD

[0001] The present application relates to a method for freezing cell aggregates including neural cells.

BACKGROUND

[0002] Transplantation of dopamine-producing (DA) neurons has been considered as a promising therapeutic method for Parkinson's disease (PD) based on reports on clinical trials using fetal mesencephalic cells (Non Patent Literature 1). In addition, a method for inducing differentiation of pluripotent stem cells (PSCs) such as embryonic stem cells (ES cells or ESCs) or induced pluripotent stem cells (iPS cells or iPSCs) into dopamine-producing neurons, or progenitor cells thereof has been reported. The group of the present inventors has already reported a method for preparing dopamine-producing neurons, or dopamine-producing neuron progenitor cells from human induced pluripotent stem cells (Non Patent Literature 2). Other groups have also reported preparation of PSC-derived dopamine-producing nerves (Non Patent Literatures 3 and 4) .

[0003] For drugs having cells as active ingredients, cryopreservation of a final product is an essential element for the dissemination of cell therapies (Patent Literatures 1 to 4). Unlike cell biology research, it is preferable that cryopreserved cells, when used in clinical practice, be transplanted immediately after thawing without recovery culture. Therefore, it is important for frozen cells to maintain their engraftment capacity, function or activity, and cell viability after thawing.

[0004] It has been suggested that transplantation of solid tissue induces a stronger immune response than transplantation of cell suspension because of the remaining donor blood vessels and antigen-presenting cells (Non Patent Literature 5). On the other hand, if the immune response is suppressed by isogeneic transplantation or an immunosuppressive agent, this problem is eliminated, and transplantation of ventral midbrain (VM) tissue shows dopamine-producing nerves having a higher survival rate and behavioral recovery than transplantation of cell suspension (Non Patent Literature 6). In addition, mechanical and enzymatic dissociation processes to obtain a cell suspension can alter cell properties to cause cell injury. Therefore, in clinical applications, it is desirable that cells to be transplanted should be administered as a sphere rather than a cell suspension. However, spheres are more difficult to cryopreserve than single cells.

[0005] When cryopreserved PSC-derived dopamine-producing nerves as a single cell suspension is transplanted into rat corpus striatum, the survival ratio of TH^+ cells is reduced to about 60% compared to unfrozen cells (Non Patent Literature 7). On the other hand, in most researches with human or rat ventral midbrain (VM) tissue cryopreserved, the dopamine-producing nerves *in vivo* have a survival rate reduced to less than 20% compared to unfrozen tissue (Non Patent Literatures 8 to 10). Therefore, there has been a need to develop a freezing method that can maintain the survival of a PSC-derived dopamine-producing neuron sphere.

[0006] In general, there are two cell cryopreservation methods (Non Patent Literatures 11 to 13). The slow cooling method of these methods is a method in which cells are frozen together with a low concentration of cryoprotectant (CPA) (such as 10% dimethyl sulfoxide (DMSO)) at about 1 °C/min (Patent Literature 5, Non Patent Literatures 14 and 15). On the other hand, the vitrification method is an ultra-fast cooling method in which cells are transferred into liquid nitrogen immediately after a high concentration of cryoprotectant is added (Patent Literature 6, Non Patent Literature 16). Since the vitrification method requires strict time control, its application to clinical cell production has been technically difficult (Non Patent Literature 17).

[0007] In the slow cooling method, on the other hand, ice formation begins first in the extracellular space, leading to the concentration of extracellular fluid. As a result, water is withdrawn from the cells by an osmotic gradient across the cell membrane. This dehydration of the cells avoids intracellular ice formation. However, if cells are excessively dehydrated, they are injured by the concentrated intracellular fluid and by the CPA in the cryopreservation solution. Because of the need for precise control of ice formation and cell dehydration, no clinical cryopreservation method for a sphere has been established.

Citation List

Patent Literature

[0008]

[Patent Literature 1]
JP2017-104061A
[Patent Literature 2]
WO2017/159862

[Patent Literature 3]
JP2015-521469A
[Patent Literature 4]
JP2008-501320A
[Patent Literature 5]
JP2011-103885A
[Patent Literature 6]
JP2013-110988A

Non Patent Literature

[0009]

[Non Patent Literature 1]
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[Non Patent Literature 16]
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[Non Patent Literature 17]
Nagano et al., Biomedical Research, 28(3), 153-160, 2007

SUMMARY OF INVENTION

TECHNICAL PROBLEM

[0010] An object of the present application is to provide a method for freezing cell aggregates including neural cells.

SOLUTION TO PROBLEM

[0011] As a result of intensive studies, the present inventors have found a method for freezing a cell aggregate including neural cells and have completed the present invention. More specifically, the present invention relates to the following:

[1] A method for freezing a cell aggregate including neural cells and having a three-dimensional structure, which comprises following steps (1) and (2):

(1) contacting the cell aggregate including neural cells and having a three-dimensional structure with a preservation solution at 0°C to 30°C prior to freezing to prepare a preservation solution-soaked cell aggregate; and
(2) cooling the preservation solution-soaked cell aggregate obtained in step (1) from a temperature at least about 5°C higher than the freezing point of the preservation solution to a temperature about 5°C lower than the freezing point at an average cooling speed of 2 to 7 °C/min to freeze the cell aggregate.

[2] The method according to [1], wherein the average cooling speed in step (2) is 3 to 7 °C/min.
[3] The method according to [1] or [2], wherein the cell aggregate is contacted with the preservation solution for 15 minutes to 90 minutes, preferably 15 minutes to 60 minutes in step (1).
[4] The method according to any one of [1] to [3], wherein the preservation solution has the freezing point of from -1°C to -10°C.
[5] The method according to any one of [1] to [4], wherein the preservation solution is an aqueous solution comprising 7% to 12% of dimethyl sulfoxide and/or propylene glycol, and step (2) is a step of cooling from 0 ± 5°C to -30 ± 5°C at an average cooling speed of 2 to 5 °C/min.
[6] The method according to any one of [1] to [5], wherein the average cooling speed in step (2) is 3 to 5 °C/min.
[7] The method according to any one of [1] to [6], wherein the method further comprises a following step (3):
(3) cooling the frozen cell aggregate obtained in step (2) to -50°C or lower.
[8] The method according to any one of [1] to [7], wherein the cell aggregate including neural cells is a cell aggregate including neural cells derived from pluripotent stem cells.
[9] The method according to any one of [1] to [8], wherein the cell aggregate including neural cells comprises cells that are positive for at least one of FOXA2, TH, and NURR1.
[10] The method according to [9], wherein the cell aggregate including neural cells comprises cells positive for FOXA2 and LMX1A.
[11] The method according to [9], wherein the cell aggregate including neural cells comprises cells positive for FOXA2, TH, and NURR1.
[12] The method according to any one of [1] to [8], wherein the cell aggregate including neural cells comprises cells positive for FOXA2 and LMX1A in an amount of 40% or more of the total cells and cells positive for TH and NURR1 in an amount of 40% or less of the total cells.
[13] The method according to any one of [1] to [12], wherein the cell aggregate including neural cells comprises a dopamine-producing neuron progenitor cell and/or a dopamine-producing neuron.
[14] The method according to any one of [1] to [13], wherein the cell aggregate includes 500 to 150000 cells.
[15] The method according to any one of [1] to [14], wherein number of cells contained in the preservation solution is 80000 to 5000000 cells/mL, and the cell aggregate has an equivalent spherical diameter of 150 to 1000 μm.
[16] The method according to any one of [1] to [15], wherein the cell aggregate and the preservation solution have volume of 0.25 mL to 2 mL.
[17] The method according to any one of [1] to [16], wherein the cell aggregate and the preservation solution are packed in a 0.5 mL to 15 mL container.
[18] A method for preserving a cell aggregate including neural cells and having a three-dimensional structure for a long-term, wherein the method comprises holding the frozen cell aggregate obtained by the method according to any one of [1] to [17] at or below -80°C.
[19] The method according to any one of [1] to [18], wherein the obtained frozen cell aggregate can be used without recovery culture after thawing.
[20] A composition for transplantation, wherein the composition comprises, as an active ingredient, the cell aggregate frozen or preserved for a long-term by the method according to any one of [1] to [19].
[21] A frozen composition for transplantation, wherein the composition comprises:

a cell aggregate including 60% or more of dopamine-producing neuron progenitor cells and dopamine-producing neurons derived from a pluripotent stem cell, having an equivalent spherical diameter of 150 μm to 1000 μm and including 500 to 150000 cells; and
a cryopreservation solution comprising 7% to 12% of dimethyl sulfoxide or propylene glycol and having the freezing point of -1°C to -10°C, and
wherein the frozen cell aggregate has following properties:

(1) about 60% or more of the total cells are viable after thawing;
(2) the cells after thawing have a neurite extension activity of 50% or more of that before freezing; and

(3) the rates of the FOXA2-positive cells, LMX1A-positive cells, NURR1-positive cells, and TH-positive cells in the cells viable after thawing are changed within ± 10% from those in the cells before freezing.

[22] The composition for transplantation according to [20] or [21], wherein number of cells in the composition is 80000 to 5000000 cells/mL, and the composition comprises cells positive for FOXA2 and LMX1A in an amount of 40% or more of the total cells and cells positive for TH and NURR1 in an amount of 40% or less of the total cells.
[23] The composition for transplantation according to any one of [20] to [22], wherein the cell aggregate has an equivalent spherical diameter of 150 to 1000 μm.
[24] The composition for transplantation according to any one of [20] to [23], wherein the composition can be used without recovery culture after thawing.
[25] The composition for transplantation according to any one of [20] to [24], wherein the composition comprises 8 to 192 cell aggregates/mL, the cell aggregate has an equivalent spherical diameter of 150 μm to 1000 μm, and number of cells per container is 80000 to 2400000.
[26] The composition for transplantation according to any one of [20] to [25], wherein the cell aggregate and the preservation solution have volume of 0.25 mL to 2 mL.
[27] The composition for transplantation according to any one of [20] to [26], wherein the composition is packed in a 0.5 mL to 15 mL container.
[28] A method for producing a composition for transplantation containing a dopamine-producing neuron progenitor cell as an active ingredient, which comprises:
freezing a cell aggregate by the method according to any one of [1] to [17], wherein number of cells in the composition is 80000 to 5000000 cells/mL, the cell aggregate comprises cells positive for FOXA2 and LMX1A in an amount of 40% or more of the total cells and cells positive for TH and NURR1 in an amount of 40% or less of the total cells, and the cell aggregate has an equivalent spherical diameter of 150 μm to 1000 μm.
[29] The method for producing the composition for transplantation according to [28] containing an aggregate of neural cells as an active ingredient, wherein the composition comprises a population of cell aggregates which have an equivalent spherical diameter of 150 to 1000 μm, number of cells per container is 80000 to 2400000, the cell aggregate and the preservation solution have volume of 0.25 mL to 2 mL, and the composition is packed in a 0.5 mL to 15 mL container.
[30] A method for treating a disease requiring regeneration of a dopamine-producing nerve, which comprises following steps of:

(1) thawing the composition for transplantation according to any one of [20] to [27] at 30°C to 40°C, preferably at 37°C ± 3°C; and
(2) transplanting the composition for transplantation obtained in (1) into a corpus striatum region of a patient.

[31] The method according to [30], wherein the cryopreservation solution is replaced with a dosing vehicle without culture after thawing to perform step (2).

EFFECTS OF THE INVENTION

[0012] The present application provides a method for cryopreserving a cell aggregate including neural cells. The neural cells cryopreserved by the method of the present application exhibit high cell viability and maintain functional properties.

BRIEF DESCRIPTION OF DRAWINGS

[0013]

[Figure 1] A scheme showing a protocol for inducing differentiation from iPSCs into dopamine-producing neuron progenitor cells and timings for evaluations. Abbreviations in the figure indicate components added to medium. LDN: LDN193189, A: A83-01, Y: Y-27632, Pur: Purmorphamine, CHIR: CHIR99021, AA: ascorbic acid.
[Figure 2A] The effect of cryopreservation solutions on iPSC-derived dopamine-producing neuron progenitor cells. Recovery of viable cells after thawing from unsorted cells that were immersed in the cryopreservation solutions shown in Table 1 for 15 minutes and then cryopreserved at 0.5 °C/min (n = 4).
[Figure 2B] The effect of cryopreservation solutions on iPSC-derived dopamine-producing neuron progenitor cells. Neurite extension of spheres derived from the unsorted cells that were immersed in the cryopreservation solutions shown in Table 1 for 15 minutes and then cryopreserved at 0.5 °C/min (n = 4) .
[Figure 2C] The effect of a cryopreservation solutions on iPSC-derived dopamine-producing neuron progenitor cells. Shown is immunostained images obtained by staining, with an early neuronal marker (PSA-NCAM), neurites of

spheres derived from unsorted cells that were immersed in the cryopreservation solutions shown in Table 1 for 15 minutes and then cryopreserved at 0.5 °C/min. The scale bar indicates 1 mm.

[Figure 3] Time-temperature curves for a sample (straight line), a freezing chamber (dashed line), and a program (dotted line). Bambanker hRM was used as the sample. The lower graph shows an enlargement of the temperature change caused by latent heat release in the upper graph.

[Figure 4A] The effect of cryopreservation programs on iPSC-derived dopamine-producing neuron progenitor cells. Recovery of viable cells after thawing from unsorted cells that were immersed in Bambanker hRM for 15 minutes and then cryopreserved according to different freezing programs.

[Figure 4B] The effect of a cryopreservation programs on iPSC-derived dopamine-producing neuron progenitor cells. Neurite extension of spheres derived from the unsorted cells that were immersed in Bambanker hRM for 15 minutes and then cryopreserved according to different freezing programs.

[Figure 5A] The effect of freezing iPSC-derived dopamine-producing neuron progenitor cells after prolonged exposure to the cryopreservation solutions. Recovery of viable cells after thawing in unsorted cells that were exposed to Bambanker hRM for 60 minutes and then cryopreserved in the different freezing programs.

[Figure 5B] The effect of freezing iPSC-derived dopamine-producing neuron progenitor cells after prolonged exposure to the cryopreservation solutions. Neurite extension of spheres derived from the unsorted cells that were exposed to Bambanker hRM for 60 minutes and then cryopreserved in the different freezing programs.

[Figure 6A] *In vitro* characteristics of the cryopreserved sphere. Recovery of viable cells after thawing.

[Figure 6B] *In vitro* characteristics of the cryopreserved sphere. Neurite extension of the sphere.

[Figure 6C] *In vitro* characteristics of the cryopreserved sphere. Immunostaining of the sphere at day 35 and day 7 after thawing. The left panels show immunostaining images for FOXA2/DAPI, the center panels show immunostaining images for NURR1/TH, and the right panels show immunostaining images for SOX1/KI67/PAX6/DAPI. The scale bar indicates 100 μm.

[Figure 6D] *In vitro* characteristics of the cryopreserved sphere. Percentages of $FOXA2^+$ cells, $NURR1^+$ cells, and TH^+ cells to total cells at day 35 and day 7 after thawing.

[Figure 6E] *In vitro* characteristics of the cryopreserved sphere. Percentages of $SOX1^+$ cells, $PAX6^+$ cells, and $KI67^+$ cells to total cells at day 35 and day 7 after thawing.

[Figure 6F] *In vitro* characteristics of the cryopreserved sphere. The gene expression of the spheres relative to GAPDH measured by quantitative RT-PCR. The expression level of undifferentiated cells (day 0) was set to 1.

[Figure 6G] *In vitro* characteristics of the cryopreserved sphere. The gene expression of the spheres relative to GAPDH measured by quantitative RT-PCR. The expression level of undifferentiated cells (day 0) was set to 1.

[Figure 6H] *In vitro* characteristics of the cryopreserved sphere. A principal component analysis of the microarray data. Temporal changes in gene expressions of unfrozen cells (circle) and frozen cells (triangle) are shown.

[Figure 6I] *In vitro* characteristics of the cryopreserved sphere. Scatter plots of the microarray data from unfrozen cells and frozen cells in the same lot at day 35 (X-axis) or day 7 after thawing (Y-axis) are shown. A solid black circle indicates a gene showing a signal intensity of 50 or more in either one of the samples, and a white circle indicates a gene showing a signal intensity of 50 or less in both samples.

[Figure 6J] *In vitro* characteristics of the cryopreserved sphere. Scatter plots of microarray data from unfrozen cells in different lots at day 35 are shown. A solid black circle indicates a gene showing a signal intensity of 50 or more in either one of the samples, and a white circle indicates a gene showing a signal intensity of 50 or less in both samples.

[Figure 6K] *In vitro* characteristics of the cryopreserved sphere. Immunostaining images for TUBB3, TH, and DAPI in iPSC-derived dopamine-producing neurons at day 28 + 21 after thawing. The scale bar indicates 50 μm.

[Figure 6L] *In vitro* characteristics of the cryopreserved sphere. A representative induced action potential of iPSC-derived dopamine-producing neurons at day 28 + 21 after thawing.

[Figure 6M] *In vitro* characteristics of the cryopreserved sphere. Results of dopamine release induced by high potassium stimulation at day 56 or day 28 + 28 after thawing.

[Figure 7A] Temporal change in the expression of a dopamine-producing neuron progenitor cell marker after thawing. Percentage of $FOXA2^+$ cells to total cells at days 28, 29, 31, and 35 and days 0, 1, 3, and 7 after thawing.

[Figure 7B] Temporal change in the expression of a dopamine-producing neuron progenitor cell marker after thawing. Percentage of $NURR1^+$ cells to total cells at days 28, 29, 31, and 35 and days 0, 1, 3, and 7 after thawing.

[Figure 7C] Temporal change in the expression of a dopamine-producing neuron progenitor cell marker after thawing. The gene expression for TH of the sphere relative to GAPDH measured by quantitative RT-PCR. The expression level of undifferentiated cells (day 0) was set to 1.

[Figure 8A] The graft survival rate and function of a cryopreserved sphere. Methamphetamine-induced rotational movements of rats having grafts. Data are presented as the mean ± SEM (n = 6 to 8). Two-way ANOVA with Tukey's multiple comparison test was performed, and significance levels are indicated by **p <0.01, ****p <0.001 relative to a solvent-administered group.

[Figure 8B] The graft survival rate and function of a cryopreserved sphere. Immunostaining images for HNA of

representative grafts derived from unfrozen cells (upper panel) and frozen cells (lower panel).
[Figure 8C] The graft survival rate and function of a cryopreserved sphere. Number of survived HNA^+ cells in grafts.
[Figure 8D] The graft survival rate and function of a cryopreserved sphere. A DAB-staining image for TH of a representative graft derived from unfrozen cells.
[Figure 8E] The graft survival rate and function of a cryopreserved sphere. A magnified image within the frame of Figure 8D. The right panel is a magnified image within the frame of the left panel. The scale bar indicates 200 μm.
[Figure 8F] The graft survival rate and function of a cryopreserved sphere. A DAB-staining image for TH of a representative graft derived from frozen cells.
[Figure 8G] The graft survival rate and function of a cryopreserved sphere. A magnified image within the frame of Figure 8F. The right panel is a magnified image within the frame of the left panel. The scale bar indicates 200 μm.
[Figure 8H] The graft survival rate and function of a cryopreserved sphere. Number of survived TH^+ cells in grafts.
[Figure 8I] The graft survival rate and function of a cryopreserved sphere. Immunostaining images for FOXA2, TH, and HNA (upper), and KI67 and HNA (lower) of grafts derived from frozen cells. The scale bar indicates 50 μm.
[Figure 8J] The graft survival rate and function of a cryopreserved sphere. Percentages of $FOXA2^+$ cells relative to HNA^+ cells.
[Figure 8K] The graft survival rate and function of a cryopreserved sphere. Percentages of $KI67^+$ cells relative to HNA^+ cells.
[Figure 9] The survival rate and neurite extension activity of cryopreserved spheres thawed under different conditions.
[Figure 10A] The expression of markers in a sphere derived from cells not sorted with anti-CORIN antibodies. Immunostaining images for LMX1A, FOXA2, and DAPI (upper), NURR1, TH, and DAPI (center), and SOX1, KI67, PAX6, and DAPI (lower) of the sphere. The scale bar indicates 100 μm.
[Figure 10B] The expression of markers in a sphere derived from cells not sorted with anti-CORIN antibodies. Percentages of $FOXA2^+$/$LMXIA^+$ cells, $NURR1^+$ cells, and TH^+ cells relative to total cells.
[Figure 10C] The expression of markers in a sphere derived from cells not sorted with anti-CORIN antibodies. Percentages of SOX1+ cells, $PAX6^+$ cells, and $KI67^+$ cells relative to total cells.
[Figure 11] The expression of markers in a sphere immediately before freezing (day 28) in the same lot as the sphere shown in Figure 6C. Immunostaining images for LMX1A, FOXA2, and DAPI (upper), NURR1, FOXA2, TH, and DAPI (center), and SOX1, KI67, PAX6, and DAPI (upper) of spheres. The scale bar indicates 100 μm.

DESCRIPTION OF EMBODIMENTS

[0014] In the present specification and claims, when a numerical value is accompanied by the term "about," it is intended to include a range of ± 10% of the value. For example, "about 20" shall include "18 to 22". A numerical range includes all numerical values between the two endpoints and the numerical values at both endpoints. The term "about" for a range applies to both endpoints of the range. Thus, for example, "about 20 to 30" shall include "18 to 33".

[Neural cells]

[0015] The present application provides a method for freezing a cell aggregate including neural cells and having a three-dimensional structure.

[0016] The neural cells include neuronal cells or neurons, progenitor cells of the neurons, i.e., neural progenitor cells or neural precursor cells.

[0017] The neural cells may be neural cells derived from any site such as neural cells of the central nervous system, or neural cells of the peripheral nervous system such as somatic neural cells of motor nerves or sensory systems or neural cells of autonomic nerves; examples of the neural cells include nerves (neurons), neural crest-derived cells, glia cells such as oligodendrocytes or astrocytes, and stem cells or progenitor cells thereof. Examples of the neural cell include a cell expressing a neural cell marker. Examples of the neural cell marker include, but are not limited to, NCAM, βIII-Tubulin (TUJ1), tyrosine hydroxylase (TH), serotonin, nestin, MAP2, MAP2AB, NEUN, GABA, glutamate, CHAT, SOX1, BF1, EMX1, VGLUT1, PAX, NKX, GSH, Telencephalin, GLUR1, CAMKII, CTIP2, TBR1, Reelin, TBR1, BRN2, OTX2, LMX1A, LMX1B, EN1, NURR1, PITX3, DAT, GIRK2, and TH. The neural cell can be identified by the expression of one or more neural cell markers. In the present specification, examples of the neural cell include a cell expressing one or more, two or more, or three or more of the above neural cell markers.

[0018] Neural cells of the central nervous system can be classified according to differences in sites where the neural cells nervous systems are present. That is, examples of the neural cells of the central nervous system include neurons derived from prosencephalon, telencephalon, diencephalon, cerebral, hypothalamus, mesencephalon, metencephalon, mesencephalon-metencephalon boundary region, cerebellum, retina, pituitary gland, or spinal cord, and progenitor cells thereof.

[0019] The neurons derived from prosencephalon are neurons that are present in prosencephalon tissue (i.e., telen-

cephalon, cerebral, hippocampus or choroid plexus, diencephalon, hypothalamus, etc.). The neurons of prosencephalon can be identified by the expression of a prosencephalon neuron marker. Examples of the prosencephalon neuron marker include OTX1 (prosencephalon), BF1 (also referred to as FOXG1) or SIX3 (also serving as a marker for telencephalon or cerebral). In the present specification, examples of the neural cell include a cell expressing one or more, two or more, or three or more of the above prosencephalon neuron markers or the markers for telencephalon or cerebral.

[0020] Examples of the neuron derived from cerebral include dorsal cells (e.g., cerebral cortex cells, Cajal-Retzius cells, hippocampus neurons) and ventral cells (e.g., basal ganglia). Examples of the ventral cerebral neuron marker include basal ganglia neuron markers (e.g., GSH2, MASH1, NKX2.1, NOZ1). Examples of the dorsal cerebral neuron marker include cerebral cortex neuron markers (e.g., PAX6, EMX1, TBR1). In the present specification, examples of the neural cell include a cell expressing one or more, two or more, or three or more of the above cerebral neuron markers, the basal ganglia neuron markers or the cerebral cortex neuron markers.

[0021] Examples of the neural cell derived from mesencephalon include neural progenitor cells derived from ventral mesencephalon, dopamine-producing neurons (also referred to as dopaminergic neurons) and dopamine-producing neuron progenitor cells (also referred to as dopaminergic neuron progenitor cells or dopaminergic progenitors). Examples of a mesencephalon-derived neural cell marker include FOXA2, EN2, and TUJ1. Examples of a FOXA2-positive and TUJ1-positive neural cell include dopamine-producing neuron progenitor cells and dopamine-producing neurons. The dopamine-producing neurons can be identified by being FOXA2-positive, NURR1-positive, and TH-positive.

[0022] The dopamine-producing neuron progenitor cells can be identified by being FOXA2-positive and LMX1A-positive. The dopamine-producing neuron progenitor cells more preferably contain cells that are positive for one or more of OTX2, LMX1A, LMX1B, CORIN, SHH, AADC, βIII-Tubulin, EN1, NURR1, PITX3, DAT, GIRK2, and TH. In the present specification, a cell aggregate including dopamine-producing neuron progenitor cells, unless otherwise specified, may comprise dopamine-producing neurons or dopaminergic neurons.

[0023] In the present specification, examples of the neural cell include a cell expressing one or more, two or more, or three or more of the mesencephalon-derived neural cell markers, the dopamine-producing neuron progenitor cell markers or the dopamine-producing neuron markers.

[0024] In the present specification, examples of the dopamine-producing neuron progenitor cells include cells expressing FOXA2 and/or LMX1A (FOXA2-positive and/or LMX1A-positive), preferably cells expressing one or more, two or more, or three or more selected from the group consisting of OTX2, LMX1B, CORIN, SHH, AADC, and βIII-Tubulin in addition to FOXA2 and LMXA1.

[0025] In the present specification, examples of the dopamine-producing neurons (dopaminergic neurons) include cells expressing TH and/or NURR1 (TH-positive and/or NURR1-positive), preferably cells expressing one or more, two or more, or three or more selected from the group consisting of FOXA2, AADC, DAT, and GIRK2 in addition to TH and NURR1.

[0026] Examples of the neurons derived from the mesencephalon-metencephalon boundary region include neurons that are present in the cerebellum, cerebellar plate tissue, ventricular zone, rhombic lip, etc. Examples of a mesencephalon-metencephalon boundary region marker include EN2 (mesencephalon), GBX2 (metencephalon), N-Cadherin (neural progenitor cells in the mesencephalon-metencephalon boundary region). Examples of a cerebellar neural progenitor cell marker include KIRREL2, PTF1A, and SOX2, which are GABAergic progenitor cell markers, and ATOH1 and BARHL1, which are cerebellar granule cell progenitor cell markers. In the present specification, examples of the neural cell include a cell expressing one or more, two or more, or three or more of the above mesencephalon-metencephalon boundary region markers, the cerebellar neural progenitor cell markers, the GABAergic progenitor cell markers, or the cerebellar granule cell progenitor cell markers.

[0027] Examples of a neural cell derived from a retina include photoreceptor cells, photoreceptor progenitor cells, retinal pigment epithelium cells, and cornea cells.

[0028] The neural cells can also be classified according to differences in neurotransmitters produced (secreted); examples thereof include dopamine-producing neurons, dopamine-producing neuron progenitor cells, GABAergic neurons, GABAergic progenitor cells, cholinergic neurons, cholinergic progenitor cells, serotonergic neurons, serotonergic progenitor cells, glutamatergic neurons, glutamatergic progenitor cells, noradrenergic neurons, noradrenergic progenitor cells, adrenergic neurons, and adrenergic progenitor cells.

[0029] Examples of the neural cells of motor nerves or sensory systems include cholinergic neurons and progenitor cells thereof.

[0030] Examples of the neural cells of autonomic nerves include cholinergic neurons, adrenergic neurons, and progenitor cells thereof.

[0031] Preferred examples of the neural cells in the present specification include dopamine-producing neurons (dopaminergic neurons), and dopamine-producing neuron progenitor cells (dopaminergic neuron progenitor cells).

[0032] Neural cells derived from a living body are cells isolated from a mammal such as humans, and examples of the cells isolated from human brain tissue include cells contained in the fetal mesencephalon tissue as described in Nature Neuroscience, 2, 1137 (1999) or N. Engl. J. Med.; 344: 710-9 (2001).

[0033] The neural cells may also be cells obtained by inducing differentiation from pluripotent stem cells such as embryonic stem cells (ES cells) and iPS cells. Examples of a method for inducing differentiation from pluripotent stem cells into neural cells include the methods described in Non Patent Literatures 3 and 4 and WO2015/034012 (dopamine-producing neuron progenitor cells), WO2009/148170 (cerebral neural cells and the like), WO2013/065763, WO2016/013669 or WO2017/126551 (hypophysial or subthalamic neural cells), WO2016/039317 (cerebellar neural cells), WO2015/076388 (telencephalic neural cells), Numasawa-Kuroiwa, Y et al., Stem Cell Reports, 2: 648-661 (2014) (neural progenitor cells), Qiu, L et al., Stem Cells Transl Med. 6 (9): 1803-1814 (2017) (dopamine-producing neuron progenitor cells).

[0034] The neural cells may be cells obtained by inducing differentiation from multipotent stem cells such as mesenchymal stem cells (MSCs). Examples of a method for inducing differentiation from mesenchymal stem cells into neural cells include the method described in J Chem Neuroanat. 96: 126-133 (2019).

[Pluripotent stem cell]

[0035] A pluripotent stem cell refers to a stem cell that has both pluripotency that enables differentiation into almost all cells existing in a living body and proliferative capacity. Pluripotent stem cells can be derived from fertilized ova, cloned embryos, germ stem cells, interstitial stem cells, or somatic cells. Examples of the pluripotent stem cell include, but are not particularly limited to, embryonic stem (ES) cells, nuclear transfer embryonic stem (ntES) cells derived from cloned embryos, spermatogonial stem cells (GS cells), embryonic germ cells (EG cells), induced pluripotent stem (iPS) cells, pluripotent stem cells derived from cultured fibroblasts and bone marrow stem cells (Muse cells). The pluripotent stem cells may be ES cells, ntES cells, or iPS cells. The pluripotent stem cells may be iPS cells in view of ethical considerations. Note that the embryonic stem cells are established from embryos within 14 days of fertilization.

[0036] Embryonic stem cells were first established in 1981 and have been applied to the production of knockout mice since 1989. Human embryonic stem cells were established in 1998 and are being used in regenerative medicine. Embryonic stem cells can be produced by culturing an inner cell mass on feeder cells or in medium containing leukemia inhibitory factor (LIF). Methods for producing embryonic stem cells are described, for example, in WO96/22362, WO02/101057, US5,843,780, US6,200,806, US6,280,718, etc. Embryonic stem cells can be obtained from a given institution or purchased commercially. For example, human embryonic stem cells KhES-1, KhES-2, and KhES-3 are available from Institute for Frontier Life and Medical Sciences, Kyoto University. Human embryonic stem cell line Rx::GFP (derived from the KhES-1 line) is available from Institute of Physical and Chemical Research. Mouse embryonic stem cell lines, EB5 and D3, are available from Institute of Physical and Chemical Research and ATCC, respectively.

[0037] A nuclear transfer embryonic stem cell (ntES cell), which is one type of embryonic stem cell, can be established from a cloned embryo created by transferring the nucleus of a somatic cell into an ovum from which the nucleus has been removed.

[0038] EG cells can be produced by culturing primordial germ cells in medium containing mSCF, LIF, and bFGF (Cell, 70: 841-847, 1992).

[0039] The term "induced pluripotent stem cell" as used herein refers to a cell obtained by reprogramming a somatic cell to have pluripotency induced using a known method. Specific examples thereof include cells obtained by reprogramming differentiated somatic cells such as fibroblasts or peripheral blood mononuclear cells by expression of any combination of a plurality of genes selected from a reprogramming gene group including OCT3/4, SOX2, KLF4, MYC (c-MYC, N-MYC, L-MYC), GLIS1, NANOG, SALL4, LIN28, ESRRB to induce pluripotency. Preferred examples of the combination of reprogramming factors include (1) OCT3/4, SOX2, KLF4, and MYC (c-MYC or L-MYC), (2) OCT3/4, SOX2, KLF4, LIN28, and L-MYC (Stem Cells, 2013; 31: 458 to 466), and (3) OCT3/4, SOX2, NANOG, and LIN28 (Science 2007; 318: 1917 to 1920).

[0040] Induced pluripotent stem cells were established with mouse cells by Yamanaka et al. in 2006 (Cell, 2006, 126 (4), pp. 663 to 676). Induced pluripotent stem cells were also established with human fibroblasts in 2007 and have pluripotency and replication competence similar to embryonic stem cells (Cell, 2007, 131 (5), pp. 861 to 872; Science, 2007, 318 (5858), pp. 1917 to 1920; Nat. Biotechnol., 2008, 26 (1), pp. 101 to 106).

[0041] Induced pluripotent stem cells can also be produced by a method of deriving induced pluripotent stem cells from somatic cells through the addition of a compound or the like, in addition to a method of producing induced pluripotent stem cells by reprogramming directly through gene expression (Science, 2013, 341, pp. 651 to 654).

[0042] It is also possible to obtain an established induced pluripotent stem cell line; for example, human induced pluripotent stem cell lines such as 201B7 cells, 201B7-Ff cells, 253G1 cells, 253G4 cells, 1201C1 cells, 1205D1 cells, 1210B2 cells, 1231A3 cells, etc. established at Kyoto University are available from Kyoto University. For example, Ff-I01 cells, Ff-I01s04 cells, QHJ-I01 and Ff-I14 cells established at Kyoto University are available from Kyoto University as the established induced pluripotent stem cell line.

[0043] Examples of somatic cells used in the production of induced pluripotent stem cells include, but are not particularly limited, tissue-derived fibroblasts, erythroid cells (e.g., peripheral blood mononuclear cells (PBMCs), T cells), hepato-

cytes, pancreatic cells, enterocytes, and smooth muscle myocytes.

[0044] For reprogramming through expression of several genes in the production of induced pluripotent stem cells, the means for expressing the genes is not particularly limited. Examples of the above-mentioned means include infection with a viral vector (e.g., retroviral vectors, lentiviral vectors, Sendai viral vectors, adenoviral vectors, or adeno-associated virus vectors), gene transfer (e.g., calcium phosphate transfection, lipofection, RetroNectin method, or electroporation) using a plasmid vector (e.g., plasmid vectors or episomal vectors), gene transfer (e.g., calcium phosphate transfection, lipofection, or electroporation) using an RNA vector, and direct injection (e.g., needle-based method, lipofection, or electroporation) of a protein.

[0045] Induced pluripotent stem cells can be produced in the presence of feeder cells or in the absence of feeder cells (feeder-free). In the production of induced pluripotent stem cells in the presence of feeder cells, the induced pluripotent stem cells can be produced in the presence of an undifferentiated-state maintenance factor by a known method. Medium used to produce induced pluripotent stem cells in the absence of feeder cells is not particularly limited and can be any known maintenance medium for embryonic stem cells and/or induced pluripotent stem cells, or any medium for establishing induced pluripotent stem cells in a feeder-free manner. Examples of the medium for establishing induced pluripotent stem cells in a feeder-free manner include feeder-free media such as Essential 8 medium (E8 medium), Essential 6 medium, TeSR medium, mTeSR medium, mTeSR-E8 medium, Stabilized Essential 8 medium, and StemFit medium. In the production of induced pluripotent stem cells, for example, four factors, OCT3/4, SOX2, KLF4, and MYC (L-MYC or C-MYC) can be transferred into somatic cells in a feeder-free manner using Sendai viral vectors . to prepare the induced pluripotent stem cells.

[0046] Pluripotent stem cells used in the present invention are mammalian pluripotent stem cells, preferably rodent (e.g., mouse or rat) or primate (e.g., human or monkey) pluripotent stem cells, more preferably human or mouse pluripotent stem cells, even more preferably human induced pluripotent stem cells (iPS cells) or human embryonic stem cells (ES cells).

[Cell aggregate]

[0047] The cell aggregate "having a three-dimensional structure" herein refers to a cell aggregate (cell aggregate or sphere) which is a three-dimensional cell population formed by cultured cells adhering to each other through, for example, a suspension culture or a 3D culture. A cell aggregate of neural cells is also referred to as a neurosphere. The shape of the cell aggregate is not particularly limited and may be spherical or non-spherical. The cell aggregate in the present specification is preferably a cell aggregate having a three-dimensional shape similar to a sphere. The three-dimensional shape similar to a sphere is a shape having a three-dimensional structure and shows, for example, a circular shape or an elliptical shape when projected onto a two-dimensional plane.

[0048] The cell aggregate including neural cells and having a three-dimensional structure has no particular restrictions on its size but usually has an equivalent spherical diameter of 150 μm to 1000 μm, and for example, 200 μm to 800 μm or 300 μm to 500 μm in one embodiment. The cell aggregate including neural cells and having a three dimensional structure usually includes 500 to 150000 cells, and in one embodiment, for example, 1000 to 100000 cells, 1000 to 70000 cells, or 3000 to 30000 cells.

[0049] The cell aggregate including neural cells may comprise other cells together with the neural cells. Examples of such a cell aggregate include a cell aggregate including 60% or more, 70% or more, 80% or more, and more preferably 90% or more of neural cells.

[0050] In one embodiment, the cell aggregate including neural cells may comprise 60% or more, 70% or more, or 80% or more of dopamine-producing neuron progenitor cells and/or dopamine-producing neurons. That is, the cell aggregate including neural cells may comprise neural cells expressing one or more markers selected from FOXA2, LMX1A, LMX1B, NURR1, and TH in an amount of 60% or more, 70% or more, or 80% or more.

[0051] In one embodiment, the cell aggregate including neural cells comprises 40% or more, 60% or more, 70% or more, 80% or more, 85% or more, or 90% or more of dopamine-producing neuron progenitor cells.

[0052] In one embodiment, the cell aggregate including neural cells comprises cells expressing one or more, two or more, or three or more markers for dopamine-producing neuron progenitor cells in an amount of 40% or more, 60% or more, 70% or more, 80% or more, 85% or more, or 90% or more.

[0053] In one embodiment, the cell aggregate including neural cells comprises cells positive for FOXA2 and LMX1A in an amount of 40% or more, 60% or more, 70% or more, 80% or more, 85% or more, or 90% or more. In one embodiment, the cell aggregate further comprises cells positive for TH and NURR1 in an amount of 40% or less.

[0054] In one embodiment, the cell aggregate including neural cells may comprise cells positive for FOXA2, TH, and NURR1 in an amount of 0% or more, 10% or more, or 20% or more.

[0055] In one embodiment, the cell aggregate including dopamine-producing neuron progenitor cells may comprise NURR1-positive cells in an amount of 60% or less, 50% or less, 40% or less, 5 to 50%, 5 to 40%, or 5 to 20%.

[0056] In one embodiment, the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-

producing neurons may comprise TH-positive cells in an amount of 30% or less, 20% or less, 1 to 30%, 5 to 30%, 1 to 20%, 5 to 20%, or 5 to 15%.

[0057] In one embodiment, the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons may comprise KI67-positive cells in an amount of 30% or less, 1 to 25%, 1 to 20%, or 5 to 20%.

[0058] In one embodiment, the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons may comprise SOX1-positive cells in an amount of 20% or less, 10% or less, 5% or less, or 1% or less.

[0059] In one embodiment, the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons may comprise PAX6-positive cells in an amount of 5% or less, 2% or less, 1% or less, or 0.5% or less.

[0060] In one embodiment, the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons further comprises cells positive for TH and NURR1 in an amount of 20% or less, specifically 1% to 20%, more specifically 5% to 15%.

[0061] In one embodiment, the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons comprises cells positive for FOXA2 and LMX1A in an amount of 50% or more, preferably 60% or more, 70% or more, or 80% or more, and cells positive for TH and NURR1 in an amount of 20% or less, 1% to 20%, more specifically 5% to 15%.

[0062] In one embodiment, in the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons, SOX1-positive cells are 10% or less, preferably 7% or less, more preferably 3% or less, and PAX6-positive cells are 5% or less, preferably 4% or less, more preferably 2% or less.

[0063] In one embodiment, the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons comprises cells positive for FOXA2 and LMX1A in an amount of 60% or more and cells positive for TH and NURR1 in an amount of 1% to 20%, and SOX1-positive cells are 10% or less, preferably 7% or less, more preferably 3% or less, and PAX6-positive cells are 5% or less, preferably 4% or less, more preferably 2% or less.

[0064] In one embodiment, the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons comprises cells positive for FOXA2 and LMX1A in an amount of 60% or more of the total cells and may comprise cells positive for TH and NURR1 in an amount of 20% or less, 1 to 20%, or 5 to 15% of the total cells.

[0065] In one embodiment, the above cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons is a cell aggregate having an equivalent spherical diameter of 150 μm to 1000 μm.

[0066] In one embodiment, the above cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons comprises cells positive for FOXA2 and LMX1A in an amount of 60% or more and cells positive for NURR1 and TH in an amount of 1% to 20% and has an equivalent spherical diameter of 150 μm to 1000 μm.

[Freezing method]

[0067] The method of the present application comprises the step of (1) contacting a cell aggregate including neural cells and having a three-dimensional structure with a preservation solution at 0°C to 30°C prior to freezing to prepare a preservation solution-soaked cell aggregate.

[0068] In the present application, a cryopreservation solution (preservation solution) refers to an aqueous solution comprising a cryoprotectant. The cryoprotectant refers to a substance that have a high affinity with water molecules and are highly effective in inhibiting the growth of ice crystals in the cryopreservation solution. Cryoprotectants include, for example, dimethyl sulfoxide (DMSO), ethylene glycol (EG), propylene glycol (PG), 1,2-propanediol (1,2-PD), 1, 3-propanediol (1,3-PD), butylene glycol (BG), isoprene glycol (IPG), dipropylene glycol (DPG), and/or glycerin. In the present application, the cryoprotectant is preferably dimethyl sulfoxide and/or propylene glycol. A concentration of the cryoprotectant in the cryopreservation solution is usually 7 to 12%, preferably about 10%, when dimethyl sulfoxide and/or propylene glycol is used as the cryoprotectant.

[0069] As the aqueous solution, for example, physiological saline, a buffered solution such as PBS, EBSS, or HBSS, a medium for culturing cells or tissues such as DMEM, GMEM, or RPMI, serum, a serum substitute, or a mixture thereof can be used.

[0070] As the cryopreservation solution, a commercially available cryopreservation solution comprising dimethyl sulfoxide (DMSO) and/or propylene glycol as a substantial component can be used. Specific examples of the cryopreservation solution include commercially available cryopreservation solutions such as STEM-CELL BANKER (SCB; ZENOAQ), STEM-CELL BANKER DMSO free (SCB DMSO free; ZENOAQ), Bambanker hRM (BBK; NIPPON Genetics), CryoStor CS5 (CS5; BioLife Solutions), CryoStor CS10 (CS10; BioLife Solutions), and Synth-a- Freeze (SaF; Thermo Fisher Scientific). For example, it is desirable to use a cryopreservation solution (such as STEM-CELL BANKER, Bambanker hRM, CryoStor CS10, or Synth-a-Freeze) comprising 7 to 12%, preferably about 10% of dimethyl sulfoxide and/or propylene glycol.

[0071] More preferably, Bambanker hRM can be used.

[0072] In the present specification, when the cell aggregate is frozen, number of cells relative to the cryopreservation solution (cell packing density) is 80000 to 5000000 cells/mL, 100000 to 4000000 cells/mL or 200000 to 2000000 cells/mL,

300000 to 1000000 cells/mL.

[0073] In the present specification, when the cell aggregate is frozen, the cell aggregate has an equivalent spherical diameter of 150 to 1000 μm, 150 μm to 600 μm, or 300 μm to 500 μm.

[0074] In the present specification, the cell aggregate and the preservation solution have volume of 0.25 mL to 2 mL, 0.5 mL to 1.5 mL, or 0.5 mL to 1 mL.

[0075] In the present specification, the cell aggregate and the preservation solution may be packed in a 0.5 mL to 15 mL, 1 mL to 5 mL, or 1 mL to 2 mL container.

[0076] The freezing point of the cryopreservation solution in the present application is not particularly limited but is usually -1°C to -10°C, preferably -3°C to -10°C, more preferably -3°C to -6°C, even more preferably about -5°C. Examples of the cryopreservation solution in the present specification include an aqueous solution comprising 7 to 12%, preferably about 10% of dimethyl sulfoxide as a substantial component and having the freezing point of - 1°C to -10°C. Also, examples of the cryopreservation solution in the present specification include an aqueous solution comprising 7 to 12%, preferably about 10% of dimethyl sulfoxide as a substantial component and having the freezing point of -3°C to -6°C.

[0077] The temperature at which the cell aggregate including neural cells is contacted with the cryopreservation solution is usually from 0°C to 30°C, preferably from 0°C to 20°C, more preferably from 0°C to 10°C, and even more preferably from 0°C to 4°C.

[0078] The period for which the cell aggregate including neural cells is contacted with the cryopreservation solution is usually 5 minutes to 240 minutes, 5 minutes to 120 minutes, preferably 5 minutes to 60 minutes, 15 minutes to 240 minutes, 15 minutes to 180 minutes, 15 minutes to 150 minutes, preferably 15 minutes to 120 minutes, 15 minutes to 90 minutes, and more preferably 15 minutes to 60 minutes.

[0079] The method of the present application also comprises the step of (2) cooling the preservation solution-soaked cell aggregate obtained in step (1) from a temperature at least about 5°C higher than the freezing point of the preservation solution to a temperature about 5°C lower than the freezing point at an average cooling speed of 2 to 7 °C/min, 2.5 to 7 °C/min, or 3 to 7 °C/min to freeze the cell aggregate.

[0080] In the method of the present application, the preservation solution-soaked cell aggregate is cooled from a temperature at least about 5°C higher than the freezing point of the preservation solution to a temperature about 5°C lower than the freezing point at an average cooling speed of 2 to 7 °C/min, 2.5 to 7 °C/min, or 3 to 7 °C/min, preferably 2 to 5.5 °C/min, 2.5 to 5.5 °C/min, or 3 to 5.5 °C/min. In a preferred embodiment, the preservation solution-soaked cell aggregate is cooled from 0 ± 5°C to - 30°C ± 5°C at an average cooling speed of 2 to 5 °C/min, 2.5 to 5 °C/min, or 3 to 5 °C/min.

[0081] The means for cooling is not particularly limited as long as the above step is achieved; a commercially available freezer can be used, and a programmed freezer (also referred to as a controlled-rate freezer) capable of controlling the temperature may be used.

[0082] In step (2), the preservation solution-soaked cell aggregate may be exposed to an electromagnetic field and/or a magnetic field. The electromagnetic field has a frequency of, for example, about 300 kHz to about 2 MHz, preferably about 500 kHz to about 1 MHz, more preferably about 600 kHz to about 1 MHz. A frequency of the magnetic field is not particularly limited but is preferably a fixed frequency. In addition, the freezing can be performed under an electrostatic field of, for example, 10 to 2000 Gauss, preferably 50 to 1000 Gauss, and more preferably 100 to 150 Gauss.

[0083] A method for achieving the above condition is not particularly limited, and for example, a programmed freezer equipped with a device for generating an electromagnetic field and a magnetic field, which is commercially available as a proton freezer (RYOHO FREEZE SYSTEMS. CO., LTD.), can be used. That is, a device having a combination of a magnetic field (a static magnetic field (SMF)), an electromagnetic wave (an alternating electric field (AEF)), and ultra-cold air may be used. More specifically, use of an electromagnetic wave of 300 kHz to 2 Mhz can inhibit the cell aggregate from being injured by formation of ice.

[0084] The method of the present application may further comprise the step of (3) cooling the frozen cell aggregate obtained in step (2) to -50°C or lower, preferably -80°C or lower, more preferably -150°C or lower.

[0085] The means for cooling is not particularly limited, and examples thereof include deep freezers, programmed freezers, proton freezers, and use of a low-temperature medium (e.g., liquid nitrogen).

[0086] The frozen cell aggregate obtained in step (2) or (3) may be held at -80°C or lower, preferably -150°C or lower for long-term preservation.

[0087] Means for long-term preservation is not particularly limited, and examples thereof include deep freezers, programmed freezers, proton freezers, and use of a low-temperature medium (e.g., liquid nitrogen).

[0088] The frozen cell aggregate can be thawed and used as appropriate. The thawing method is not particularly limited, but it is desirable to perform the thawing at about body temperature in a short period from the viewpoint of the function, activity, and viability of the cells. Specifically, it is desirable to perform the thawing at 30°C to 40°C, preferably at 35°C to 38°C, and more preferably at a temperature around human body temperature, for example, at about 37°C.

[0089] The cell aggregate frozen by the method of the present invention may be subjected to recovery culture after thawing by replacing the cryopreservation solution with a medium or may be transplanted into a living body without

performing recovery culture.

[0090] That is, the cell aggregate frozen by the method of the present invention can maintain properties equivalent to those of an unfrozen cell aggregate. For example, the cell aggregate frozen by the method of the present invention and then subjected to recovery culture for 7 days after thawing has a marker expression rate equivalent to that of the cell aggregate before freezing. For example, in the case of the cell aggregate including dopamine-producing neuron progenitor cells, examples of markers expressed on the cells include FOXA2, LMX1A, NURR1 and TH. The equivalent marker expression rate means that the difference in numerical values of the percentages of the cells expressing a maker to the total cells between before freezing and after thawing, or after culturing for 7 days after thawing is about 10% or less.

[0091] The cell aggregate frozen by the method of the present invention is useful in that it can be transplanted into a living body without recovery culture.

[Pharmaceutical composition]

[0092] The present application further provides a pharmaceutical composition, i.e., a composition (formulation) for transplantation, comprising, as an active ingredient, the cell aggregate frozen or preserved for a long-term by the above method.

[0093] The pharmaceutical composition (composition for transplantation) of the present invention is a concept including both a pharmaceutical composition frozen by the method of the present invention and a pharmaceutical composition obtained by thawing the frozen pharmaceutical composition. That is, examples of the pharmaceutical composition (composition for transplantation) of the present invention include a frozen or unfrozen composition comprising the cell aggregate including neural cells and the cryopreservation solution, as well as a composition comprising the cell aggregate including neural cells and a dosing vehicle where the cryopreservation solution has been replaced with the dosing vehicle after thawing.

[0094] Examples of the pharmaceutical composition (composition for transplantation) of the present invention include the compositions for transplantation according to [20] to [27] above.

[0095] Also, in one embodiment, the present application provides a frozen composition for transplantation, wherein the composition comprises:

a cell aggregate including 60% or more of dopamine-producing neuron progenitor cells and dopamine-producing neurons derived from a pluripotent stem cell, having an equivalent spherical diameter of 150 μm to 1000 μm and including 500 to 150000 cells; and
a cryopreservation solution comprising 7% to 12% of dimethyl sulfoxide or propylene glycol and having the freezing point of -1°C to -10°C, the cryopreservation solution being preferably Bambanker hRM, and
wherein the frozen cell aggregate has following properties:

(1) about 60% or more of the total cells are viable after thawing;
(2) the cells after thawing have a neurite extension activity of 50% or more of that before freezing; and
(3) the rate of the FOXA2-positive cells, LMX1A-positive cells, NURR1-positive cells, and TH-positive cells in the cells viable after thawing are changed within ± 10% from those in the cells before freezing, preferably, the rate of the FOXA2-positive cells, LMX1A-positive cells, NURR1-positive cells, TH-positive cells, EN1-positive cells, and PITX3-positive cells are changed within ± 10%.

[0096] As one embodiment, the present invention encompasses a composition for transplantation wherein number of cells in the composition is 80000 to 5000000 cells/mL, 100000 to 4000000 cells/mL, or 200000 to 2000000 cells/mL, 300000 to 1000000 cells/mL, and the composition comprises FOXA2-positive and LMX1A-positive cells in an amount of 40% or more, preferably 60% or more, 60% or more, 80% or more, 85% or more, or 90% or more of the total cells, and TH-positive and NURR1-positive cells in an amount of 40% or less, 1% to 20%, or 5% to 15% of the total cells.

[0097] In one embodiment, the cell aggregate has an equivalent spherical diameter of 150 to 1000 μm, 150 μm to 600 μm, or 300 μm to 500 μm.

[0098] In one embodiment, the cell aggregate and the preservation solution have volume of 0.25 mL to 2 mL, 0.5 mL to 1.5 mL, or 0.5 mL to 1 mL.

[0099] In one embodiment, the cell aggregate and the preservation solution may be packed in a 0.5 mL to 15 mL, 0.5 mL to 5 mL, or 1 mL to 2 mL container.

[0100] As one embodiment, the present invention encompasses a composition for transplantation that can be used without recovery culture after thawing.

[0101] As one embodiment, the present invention encompasses the composition for transplantation according to any one of [20] to [24], wherein the composition comprises the cell aggregates in 8 to 192 cell aggregates/mL, the cell aggregates have an average particle size of 150 μm to 1000 μm, and number of cells per container is 80000 to 2400000.

[0102] The cell aggregate is useful as a pharmaceutical composition for transplantation for a patient suffering from a disease requiring transplantation of neural cells and can be used as a drug such as a therapeutic drug for a disease accompanied by degeneration, injury, or dysfunction of neural cells. That is, a pharmaceutical composition comprising the cell aggregate of the present invention and a pharmaceutically acceptable carrier is also within the scope of the present invention.

[0103] Examples of the disease requiring transplantation of neural cells or the disease accompanied by injury or dysfunction of neural cells include spinal cord injury, motor nerve disease, multiple sclerosis, amyotrophic lateral sclerosis, atrophic lateral sclerosis, Huntington's disease, multiple system atrophy, spinocerebellar degeneration, Alzheimer's disease, Retinitis pigmentosa, age-related macular degeneration, and parkinsonian syndrome (including Parkinson's disease).

[0104] One embodiment of the present invention includes a pharmaceutical composition for treating Parkinson's disease, comprising, as an active ingredient, the cell aggregate of the present invention including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons. Number of dopamine-producing neuron progenitor cells and/or dopamine-producing neurons included in the therapeutic agent for Parkinson's disease is not particularly limited as long as the graft can be engrafted after the administration; for example, 1.0×10^4 cells or more can be included per transplantation. In addition, number of cells may be appropriately increased or decreased according to symptoms or body size to prepare the therapeutic agent. Transplantation of the dopamine-producing neuron progenitor cells to a disease site can be performed, for example, by the technique described in Nature Neuroscience, 2, 1137 (1999) or N Engl J Med.; 344: 710 to 9 (2001).

[0105] As one embodiment, the pharmaceutical composition (also referred to as the composition for transplantation) of the present invention comprises a cell aggregate including neural cells to be transplanted into a human and a cryopreservation solution. The pharmaceutical composition of the present invention includes both a frozen solid form and a liquid form before freezing or after thawing. The pharmaceutical composition may include an additive used to maintain the survival of cells as appropriate, to such an extent that it will not affect the freezing rate and freezing temperature. Examples of the cryopreservation solution include those described above.

[0106] As described below, the pharmaceutical composition or the composition for transplantation of the present invention is used for transplantation after thawing it and removing and replacing the cryopreservation solution with a dosing vehicle injectable to a living body. That is, a composition comprising the thawed cell aggregate and the dosing vehicle is also within the scope of the pharmaceutical composition (also referred to as the composition for transplantation) of the present invention.

[A method for producing a composition for transplantation]

[0107] The pharmaceutical composition (composition for transplantation) according to [20] to [27] above can be produced by the freezing method according to any one of [1] to [17] above. That is, the present invention encompasses a method for producing the above-described pharmaceutical composition (composition for transplantation).

[Therapeutic method]

[0108] One embodiment of the present invention includes a method for treating a disease requiring supplementation of neural cells, which comprises the step of transplanting the cell aggregate of the present invention into a patient suffering from the disease requiring transplantation of neural cells.

[0109] In one embodiment of the present invention, the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons as obtained by the present invention can be administered to a patient with Parkinson's disease as a pharmaceutical composition, specifically as material for transplantation.

[0110] Specifically, the administration is performed as follows: the frozen pharmaceutical composition of the present invention comprising the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons, and the cryopreservation solution is thawed, appropriately suspended in an appropriate medium for transplantation such as physiological saline, and then transplanted into a region of a patient where dopaminergic neurons are insufficient, for example, the corpus striatum. For example, the pharmaceutical composition after thawing may be washed with a vehicle comprising an appropriate carrier, and the cryopreservation solution may be replaced with a transplantation vehicle for suspending the cell aggregate for the transplantation into a human. A thawing temperature is not particularly limited but can be 30°C to 40°C, preferably 35°C to 38°C, and more preferably a temperature around human body temperature, for example, about 37°C as described above.

[0111] The cell aggregate included in the pharmaceutical composition (composition for transplantation) of the present invention can be transplanted into a living body by replacing the cryopreservation solution with a dosing vehicle without recovery culture after thawing.

[0112] For the carrier used for the transplantation vehicle (dosing vehicle) used for the cell aggregate including

dopamine-producing neuron progenitor cells and/or dopamine-producing neurons, any substance known to those skilled in the art can be used, as long as it is a substance used to maintain survival of cells. Specifically, a physiological aqueous solvent (such as physiological saline, a buffer solution, serum-free medium, etc.) can be used. In transplantation medicine, if necessary, the pharmaceutical composition including tissue or cells to be transplanted may be combined with a preservative, a stabilizing agent, a reducing agent, or an isotonizing agent which is commonly used.

[0113] For the transplantation, the thawed cell aggregate may be preserved in a vehicle necessary to maintain the viability of each cell aggregate. Examples of the "vehicle necessary to maintain the viability" include medium and a physiological buffer solution; the vehicle is not particularly limited as long as the vehicle allows the cell population including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons to survive, and those skilled in the art can appropriately select such a vehicle. One example of the vehicle is a medium prepared by using a medium usually used for culturing animal cells as a basal medium. Examples of the basal medium include media that can be used for culturing animal cells, such as BME medium, BGJb medium, CMRL 1066 medium, GMEM medium, Improved MEM Zinc Option medium, Neurobasal medium, IMDM medium, Medium 199 medium, Eagle MEM medium, αMEM medium, DMEM medium, F-12 medium, DMEM/F12 medium, IMDM/F12 medium, Ham medium, RPMI 1640 medium, Fischer's medium or mixed media thereof.

[0114] Due to the transplantation of the above cell aggregate, the transplanted dopamine-producing neuron progenitor cells and/or dopamine-producing neurons as well as dopamine-producing neuron progenitor cells and/or dopamine-producing neurons that are induced after the transplantation are functionally engrafted in the patient to which the cell aggregate is administered.

[0115] The term "engraftment" as used herein means that transplanted cells survive in a living body for a long period of time (e.g., 30 days or more, 60 days or more, or 90 days or more) and adhere and remain in an organ.

[0116] The term "functional engraftment" as used herein means a state in which transplanted cells are engrafted to perform their original function in a living body.

[0117] The term "functional survival rate" as used herein refers to the percentage of the cells that have achieved functional engraftment in the transplanted cells. The functional survival rate of transplanted dopamine-producing neuron progenitor cells can be determined, for example, by measuring number of TH-positive cells in the graft.

[0118] Due to the transplantation of the above cell aggregate, the transplanted cells and the dopamine-producing neuron progenitor cells and/or dopamine-producing neurons induced after the transplantation have a functional survival rate of 0.1% or more, preferably 0.2% or more, more preferably 0.4% or more, even more preferably 0.5% or more, and still more preferably 0.6% or more.

[0119] As one embodiment, the present invention encompasses a method for treating a disease requiring regeneration of a dopamine-producing nerve, which comprises following steps of:

(1) thawing the composition for transplantation according to any one of [20] to [27] at 30°C to 40°C, preferably at 37°C ± 3°C; and
(2) transplanting the composition for transplantation obtained in (1) into a corpus striatum region of a patient.

[0120] One embodiment of the present invention encompasses the treating method, wherein the cryopreservation solution is replaced with a dosing vehicle without culture after thawing to perform step (2).

[0121] Examples of a mammal to undergo the transplantation in the present specification include humans, mice, rats, guinea pigs, hamsters, rabbits, cats, dogs, sheep, pigs, cows, horses, goats, and monkeys, preferably rodents (e.g., mice and rats) or primates (e.g., humans and monkeys), and more preferably humans.

EXAMPLES

[0122] Hereinafter, the present application is described in more detail with reference to Examples but is not limited to the Examples.

Example 1

[0123] An outline of Example 1 is shown in Figure 1.

[Material and method]

Maintenance and neural differentiation of human iPS cells

[0124] In accordance with the dual SMAD inhibition and floor plate induction protocol described in Doi et al., 2014, iPSC-derived dopamine-producing neuron progenitor cells were inducted. That is, human iPSCs (1231A3) (Kyoto Uni-

versity) were maintained in Stem Fit medium (AJINOMOTO CO., INC.) on a 6-well plate coated with iMatrix-511 (Nippi, Inc.). To initiate neuronal differentiation, the iPSCs were incubated with TrypLE select (Invitrogen) for 10 minutes, then dissociated into single cells and seeded with differentiation medium at a density of 5×10^6 cells/well on the 6-well plate coated with iMatrix-511 (Nippi, Inc.). The differentiation medium was medium containing GMEM supplemented with 8% KSR, 0.1 mM MEM non-essential amino acids (all from Invitrogen), sodium pyruvate (Sigma-Aldrich), and 0.1 mM 2-mercaptoethanol. The differentiation medium was changed every day from the day after seeding until day 12. To increase cell viability after seeding, 10 μM Y-27632 (WAKO CHEMICAL CO., LTD.) was added on day 1. To efficiently induce the neuronal differentiation, LDN193189 (STEMGENT, INC.) and A83-01 (WAKO CHEMICAL CO., LTD.) were added. To induce floor plate cells, 2 μM Purmorphamine and 100 ng mL^{-1} FGF8 (WAKO CHEMICAL CO., LTD.) were added on days 1 to 7, and 3 μM CHIR99021 (WAKO CHEMICAL CO., LTD.) was added on days 3 to 12 (see Figure 1).

Cell sorting and culture

[0125] On day 12, cells expressing CORIN (a floor plate marker in the developing brain) were isolated to concentrate the dopamine-producing neuron progenitor cells, and sorting (FACS technique) was performed thereon. First, cultured cells were stained with PE-labeled anti-CORIN antibody (100ng/mL; Catalent/BD) for 20 minutes. Dead cells and debris were excluded through 7-AAD staining. Analysis was performed using a FACSAria II or III cell sorter and FACSDiva software program (BD Biosciences). After cell sorting on day 12 of culture, the sorted cells were reseeded at a density of 2-3 $\times$ 10^4 cells/well in neuronal differentiation medium containing neurobasal medium supplemented with B27 supplement, 2 μM Glutamax-I (all from Invitrogen), 10 ng mL^{-1} GDNF, 200 mM ascorbic acid, 20 ng mL^{-1} BDNF (all from WAKO CHEMICAL CO., LTD.) and 400 μM dbcAMP (Sigma-Aldrich) on a low cell adhesion U-shaped 96-well plate (Sumitomo Bakelite Co., Ltd.), and cultured as floating spheres until day 28. Unsorted cells were also reseeded at a density of 1.5 $\times$ 10^4 cells/well. Half of the medium was replaced every 3 days, and 30 μM Y-27632 (WAKO CHEMICAL CO., LTD.) was added only to the first medium. For prolonged culture, the floating spheres were cultured in the neuronal differentiation medium (see Figure 1).

Cryopreservation method

[0126] The spheres collected on day 28 were cryopreserved and used in subsequent experiments. That is, the collected spheres were placed in cryovials containing 1 mL of ice-cold cryopreservation solution shown in Table 1 and kept on ice until freezing. For cryopreservation, the vials were transferred into a freezing container: BICELL (NIHON FREEZER CO., LTD.), a programmed freezer: PDF-150, 250 (STREX Inc.), cryomed (Thermo Fisher Scientific Inc.) or a proton freezer (RYOHO FREEZE SYSTEMS. CO., LTD.). Six different cooling profiles (shown in Figure 3) were used in this experiment. That is, BICELL was transferred into a deep freezer (-80°C) and kept for 4 hours or more (upper left in Figure 3). In the controlled-rate freezing method, the vials were frozen to -40°C at a rate of 0.5 °C/min (upper center in Figure 3) or 1 °C/min (upper right in Figure 3) and then cooled to -80°C at a faster rate of 3 to 5 °C/min. In the shock cooling method, the procedure of freezing to -35°C at a rate of -25°C/min and subsequent warming to -12°C at a rate of +10°C/min was inserted at a temperature of -4°C during freezing (lower left and center in Figure 3). The vials frozen with the proton freezer were kept in a chamber for 30 to 60 minutes (lower right in Figure 3). The proton freezer has a static magnetic field, electromagnetic waves, and cold air in combination. It is considered that static magnetic fields and electromagnetic fields influence the orientation of water molecules and cause the formation of small ice crystals, thereby preventing cell disruption. However, these mechanisms have not yet been fully understood. After freezing, the cryovials were preserved in the vapor phase of a liquid nitrogen tank. The frozen cells were thawed at 37°C for about 2 minutes and transferred into a 15 mL tube containing 10 mL of neurobasal medium. After removal of the supernatant, the cells were rinsed with PBS and used for each assay or transplantation. To estimate number of cells after cryopreservation, about 50 aggregates were dissociated and counted with a hemacytometer to calculate the cell concentration one day after thawing and before freezing.

[0127] Table 1 Commercially available xeno-free cryopreservation solutions used in the present Examples

[Table 1]

Medium	Brand name	Supplier	Mejor component
SCB	STEM-CELLBANKER	ZENOAQ	10 % DMSO
SCB DMSO free	STEM-CELLBANKER DMSO free	ZENOAQ	10 % Propylene glycol
BBK	Bambanker hRM	NIPPON Genetics	10 % DMSO
CS5	Cryostor CS5	BioLife Solutions	5 % DMSO
CS10	Cryostor CS10	BioLife Solutions	10 % DMSO

(continued)

Medium	Brand name	Supplier	Mejor component
SaF	Synth-a-Freeze	Thermo Fisher Scientific	10 % DMSO

Quantitative RT-PCR

[0128] Total RNA was extracted from undifferentiated cells, iPSC-derived dopamine-producing neurons (on day 28, day 29, day 31, and day 35), and iPSC-derived dopamine-producing neurons after thawing on days 28 + 0, 1, 3, and 7 using RNeasy Mini Kit or RNeasy Micro Kit (Qiagen) and cDNA was synthesized using Super Script III First-Strand Synthesis System (Invitrogen). Quantitative PCR reactions were performed by using Power SYBR Green PCR Master Mix (Applied Biosystems) with StepOne. Data were evaluated using the delta Ct method and normalized by GAPDH expression. The primer sequences used are shown in Table 2.

[Table 2]

Gene	**Forward**	**Reverse**
POU5F1	AGACCATCTGCCGCTTTGAG (SEQ ID NO: 1)	GCAAGGGCCGCAGCTT (SEQ ID NO: 2)
NANOG	GGCTCTGTTTTGCTATATCCCCTAA (SEQ ID NO: 3)	CATTACGATGCAGCAAATACGAGA (SEQ ID NO: 4)
FOXA2	TTCAGGCCCGGCTAACTCT (SEQ ID NO: 5)	AGTCTCGACCCCCACTTG CT (SEQ ID NO: 6)
LMX1A	GATCCCTTCCGACAG GGTCTC (SEQ ID NO: 7)	GGTTTCCCACTCTGGACTGC (SEQ ID NO: 8)
EN1	TGGGTGTACTGCACACGTTATTC (SEQ ID NO: 9)	GGAACTCCGCCTTGAGTCTCT (SEQ ID NO: 10)
NURR1	CGAAACCGAAGAGCCCACAGGA (SEQ ID NO: 11)	GGTCATAGCCGGGTTGGAGTCG (SEQ ID NO: 12)
PITX3	GGGCCAGGAGCACAGCGACTCA (SEQ ID NO: 13)	GCTGCCGCCGCTGCTTCTTTTT (SEQ ID NO: 14)
TH	GCAGTTCTCGCAGGACATTG (SEQ ID NO: 15)	CGGCACCATAGGCCTTCA (SEQ ID NO: 16)
TPH2	TCAGCTACTTGGCAGCTCAAC (SEQ ID NO: 17)	CTTGCCACTTTCGGTAGCAG (SEQ ID NO: 18)
GAPDH	GGTCGGAGTCAACGGATTTG (SEQ ID NO: 19)	TCAGCCTTGACGGTGCCATG (SEQ ID NO: 20)

Immunofluorescence studies

[0129] In *in vitro* studies, cultured cells on day 35 or on day 7 after thawing were fixed with 4% paraformaldehyde. In the *in vivo* studies, fixed and frozen brains were sliced into 40-μm thin slices. The slices were immunostained using the free-floating method. The primary antibodies used are shown in Table 3. Cells were visualized using a fluorescence microscope (BZ-9000; Keyence Corporation) and a confocal laser scanning microscope (Fluoview FV1000D; Olympus). Number of immunoreactive cells was quantified in every 6th sectionthroughout the grafts and corrected using the Abercrombie method.

[Table 3]

Antibody	**Species**	**Dilution**	**Supplier**	**Catalog#**
TH	Rabbit	1:400	Millipore	AB152
NURR1	Rat	1:1000	Donated by KAN labora tory	-
FOXA2	Goat	1:500	R&D systems	AH2400
SOX1	Goat	1:100	R&D systems	AH3369
PAX6	Mouse	1:500	BD Pharmingen	561462
KI67	Rabbit	1:1000	Novocastra	NCLKi67P

(continued)

Antibody	Species	Dilution	Supplier	Catalog#
TUBB3	Mouse	1:400	Covance	MMS-435P
HNA	Mouse	1:500	Millipore	MAB1281
*TUBB3 indicates βIII-Tubulin.				

Neurite extension assay

[0130] On day 28, floating spheres picked for a neurite extension assay were cultured on a 24-well plate coated with iMatrix-511 for 5 days and fixed with 4% paraformaldehyde. The spheres were stained with PE-labeled anti-PSA-NCAM antibody (1:100; Miltenyi Biotec) and visualized using a fluorescence microscope (BZ-9000; Keyence). The area of PSA-NCAM-positive neurites without cell bodies was measured using Photoshop (Adobe systems) and WinRoof (Mitani Corporation).

Electrophysiologic analysis

[0131] The suspension spheres were dissociated with papain on day 28 and cultured on a plate coated with poly-l-ornithine, fibronectin, and laminin (O/F/L) until day 49. Nerves with large cell bodies and neurite-like structures were selected for whole-cell patch-clamp recording. The cells were maintained in a physiological saline solution containing following components: 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, and 17 mM glucose. A patch pipette was prepared from a borosilicate glass capillary (GC150TF-10; Clark). This patch pipette had a resistance of 3 to 4 MW when filled with an internal solution composed of 140 mM KCl, 10 mM HEPES, and 0.2 mM EGTA (pH 7.3). Voltage-clamp and current-clamp recordings were performed using a patch-clamp amplifier (EPC-8; HEKA). The gigaseal resistances were in the range of 10 to 20 GW. The current signals from a patch clamp amplifier were filtered at 5 kHz through a 4-pole low-pass filter (UF-BL2; NF) having the Bessel characteristics, sampled using a 12-bit A/D converter, and stored on a 32-bit computer (PC-9821Ra333; NEC). All experiments were performed at room temperature.

Dopamine release assay

[0132] On day 28, floating spheres picked for a dopamine release assay were cultured on a 12-well plate coated with O/F/L for another 28 days, washed twice with a solution having a low concentration of KCl (4.7 mM), and incubated in a solution having a low concentration of KCl for 15 minutes. The medium was then replaced with a solution having a high concentration of KCl (60 mM) for 15 minutes. The solution was collected, and its dopamine concentration was determined by LC/MS/MS. The cells remaining on the plate were collected in PBS and sonicated. The DNA concentration of a cell lysate was measured using the Quant-iT ™ dsDNA Assay Kit (Thermo Fisher Scientific Inc.) and used to correct the dopamine concentration.

Microarray analysis

[0133] A cDNA microarray analysis was performed in the BioMedical Department of Kurabo Industries Ltd. The total RNA of the undifferentiated cells, the cultured cell on day 12, the iPSC-derived dopamine-producing neurons (day 28, day 29, day 31, and day 35), and the iPSC-derived dopamine-producing neurons after thawing on days 28 + 0, 1, 3, and 7 was processed using Genechip® 3'IVT pico Reagent Kit and Human Genome U133 Plus 2.0 array (Affymetrix, Inc.). Data analysis was performed using Genechip Operating Software ver. 1.4 (Affymetrix, Inc.). Signal detection and quantification was performed using MAS5 algorithm. Global normalization was performed such that the average signal intensity of all probe sets was equal to 100. Analysis was performed using data on a probe set showing a signal intensity higher than 50 at $p < 0.05$ and showing a 2-fold or more change between the samples.

Cell transplantation

[0134] Experimental animals were treated and handled in accordance with the Guidelines for Animal Experiments of Sumitomo Dainippon Pharma Co., Ltd., Guidelines for Animal Experiments of Kyoto University, and the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources (ILAR; Washington, DCA). Male F344/NJcl-rnu/rnu (nude) rats, Parkinson's disease model F344 NJcl-rnu/rnu rats (CLEA Japan, Inc.), and Sprague

Dawley (SD) rats (SHIMIZU LABORATORY SUPPLIES Co., Ltd.) were used for a short-term transplantation study. The SD rats were injected with 6-OHDA into the medial forebrain bundle in the right hemisphere at following coordinates (A, -4.0; L, -1.3; V, -7.0). A total of 19.2 mg of 6-OHDA (in 3 μL of physiological saline containing 0.02% ascorbic acid) was injected into each rat. From one day prior to transplantation, the SD rats were immunosuppressed daily with cyclosporine (10 mg/kg, i.p., LC Laboratories). Cell transplantation was performed with stereotactic injection of the spheres (A, +1.0; L, -3.0; V, -5.0 and -4.0; as well as TB, 0 (2 μl; 200,000 cells/μl).

[0135] Nude rat models for Parkinson's disease were used for a long-term transplantation study. Cell transplantation was performed with stereotactic injection of the unfrozen spheres (A, +1.0; L, -3.0; V, -5.0 and - 4.0; as well as TB, 0 (2μl; 200,000 cells/μl) or the cryopreserved spheres (A, +1.0; L, -3.5 and 2.5; V, -5.5 and -4.5; as well as TB, 0 (4μl; 200,000 cells/μl) into the corpus striatum of the right brain. The experimental animals were transcardially anesthetized and perfused with PBS followed by 4% paraformaldehyde.

Behavioral analysis

[0136] An evaluation of a methamphetamine-induced rotational movement was performed before the transplantation and every 4 weeks after the transplantation using rotation bowls monitored with a video. Methamphetamine (Sumitomo Dainippon Pharma Co., Ltd.) at a dose of 2.5 mg/kg was injected intraperitoneally and rotations were recorded for 90 minutes.

Statistical analysis

[0137] Statistical analysis was performed using a commercially-available software package (GraphPad Prism 6; GraphPad Software). Data from *in vitro* were analyzed by the one-way ANOVA and Tukey's post hoc analysis (Figures 2A, 2B, 4A, 4B, 5A, and 5B). Behavioral data were analyzed by two-way ANOVA with Tukey's multiple comparison test (Figure 8A). The differences were considered to be statistically significant for values of $p < 0.05$. The data were presented as mean ± SD, except for the behavioral data (mean ± SEM).

[Result]

(1) Examination result on cryopreservation solutions (Reference Example)

[0138] To establish the cryopreservation conditions suitable for iPSC-derived dopamine-producing neuron progenitor spheres as described above, clinically applicable cryopreservation solutions listed in Table 1 above were compared.

[0139] First, as described in Doi et al., 2014, conditions for a cryopreservation solution were screened using neural progenitor spheres that were not sorted by CORIN. It has been reported that number of cells reduces after thawing due to necrosis and subsequent persistence of delayed apoptosis and delayed necrosis (Milosevic et al., 2005). It is known that the proliferative capacity begins to recover during this process and number of cells bottoms out about 24 to 48 hours after thawing (Malpique et al., 2010; Mitchell et al., 2014; Baust et al., 2017). It has also been reported that neurotoxic compounds or diseases related to genetic aberration can cause neurite abnormality (Radio et al., 2008; Reinhardt et al., 2013; Ryan et al., 2016). These phenomena suggest that neurite morphology is a good indicator of characteristics of a neuron. Therefore, the recovery rate of viable cells one day after thawing was evaluated as cell viability, and also the area of neurite extension was examined as a new functional evaluation. The results are shown in Figure 2A, Figure 2B, and Figure 2C.

[0140] In all cryopreservation solutions, the cell viability and neurite extension significantly reduced after freezing (Figure 2). All the preservation solutions with 10% DMSO showed significantly higher viability than STEM-CELL BANKER DMSO free (SCB DMSO free) (21 ± 7%) and CryoStor CS5 (CS5) (16 ± 6%) (Figure 2A). Bambanker hRM (BBK) and Synth-a-Freeze (SaF) showed a tendency of relatively greater neurite extension than the other freezing conditions (Figure 2B and Figure 2C). 8 ± 17%, 50 ± 43%, and 13 ± 25% of the spheres did not adhere to the plate in the SCB DMSO free condition, CS5 condition, and CryoStor CS10 condition, respectively. Since BBK was already registered in the Drug Master File in Japan, BBK was used in following experiments.

(2) Effects of intermediate speed cooling and suppression of supercooling

[0141] The unsorted spheres in BBK were then cryopreserved using six different protocols. The temperature profile for each protocol is shown in Figure 3. In addition, a shock cooling method having a process of transient temperature drop (Morris and Acton., 2013) was also examined in order to verify the effect of controlling ice nucleation and latent heat at the freezing point. Proton freezers have the distinctive cooling profile in addition. to magnetic field and electromagnetic waves. Proton freezers cool a sample from -4°C to -30°C at about 5°C/min without supercooling. This cooling

rate is intermediate between the conventional slow cooling method and the vitrification method. Specifically, in the case of cooling in a proton freezer, the sample in the chamber is cooled at about 2 to 7°C/min as an actual value.

[0142] The conditions of the proton freezer and 0.5°C/min and 1°C/min without shock cooling process resulted in relatively higher cell viability than the other conditions (Figure 4A). In particular, the conditions of the proton freezer and 0.5°C/min showed about 80% cell viability compared to unfrozen cells. Also, the proton freezer showed significantly greater neurite extension than the other freezing conditions, except for the condition of 1°C/min with the shock cooling process, which was about 60% extension compared to that of the unfrozen cells (Figure 4B). These results demonstrate the maintenance of cellular functions in the condition of the proton freezer. When the time for immersion in the cryoprotectant before freezing was extended to 1 hour, good results for cell viability and neurite extension was obtained under the proton freezer condition as in the case of the immersion time of 15 minutes (Figures 5A and B). In addition, the cell viability was reduced under the condition of 0.5°C/min, but no significant differences were observed for cell viability or neurite extension under the other conditions (Figures 5A and 5B). Therefore, it was found that the intermediate speed cooling and suppression of supercooling are effective for cryopreservation of iPSC-derived dopamine-producing neuron progenitor spheres.

[0143] The spheres used above were induced in the same manner as in Figure 1, except that sorting with anti-CORIN antibody was not performed. The results of measuring the expression of the markers in the spheres before freezing are shown in Figure 10 and Table 4.

[Table 4]

	Percentage relative to DAPI-positive cells
FOXA2/LMX1A	8 4. 9 ± 5. 5 %
NURR1	2 1. 2 ± 1 1. 3 %
TH	7 . 4 ± 2 . 7 %
SOX1	4. 8 ± 2. 0%
PAX6	2. 6 ± 1. 0 %
KI67	8 . 5 ± 1. 8 %

(3) Cell number and function of cryopreserved iPSC-derived dopamine-producing neuron progenitor spheres

[0144] Based on the above screening results, the properties of the iPSC-derived dopamine-producing neuron progenitor spheres cryopreserved using BBK and the proton freezer were examined after thawing. In order to concentrate the iPSC-derived dopamine-producing neuron progenitor cells to 80% or more, the cells were sorted using CORIN antibody, and subjected to suspension culture under the above-described conditions to form cell aggregates, followed by cryopreservation using BBK and a proton freezer (see Figure 1). The immersion time in BBK was 1 hour.

[0145] As a result, the cryopreserved spheres were 200 μm to 500 μm in size, and number of cells was 5500 to 12000 (the number of cells was not measured in all spheres and is estimated to be 2000 to 15000 in view of size of the spheres). The resulting spheres showed a cell viability of 52 ± 8% and a neurite extension of 51 ± 19% (Figures 6A, B). To confirm that the spheres are dopamine-producing neuron progenitor cells, protein markers and gene expression were examined 7 days after thawing. As a result of immunocytochemistry, it was found that the cryopreserved spheres expressed almost the same level of the dopamine-producing neuron progenitor cell marker (FOXA2), the dopamine-producing nerve markers (NURR1 and TH), and the proliferating cell marker (KI67) as compared with the unfrozen spheres (Figures 6C to E).

[0146] Because the dopamine-producing neuron progenitor cells were highly concentrated by the cell sorting process, cells expressing neural stem cell markers (SOX1 and PAX6) were quite few in both unfrozen and frozen spheres. This indicates that no abnormal proliferation were occurred by the cryopreservation process. Further, a qPCR analysis showed that the expression levels of dopamine-producing neuron progenitor cell markers (FOXA2, LMX1A, and EN1) and dopamine-producing nerve markers (NURR1, PITX3, and TH) were not changed between with or without cryopreservation (Figure 6G). The expression level of pluripotency markers (POU5F1 and NANOG) was maintained less than 1% of day 0 in both spheres (Figure 6F).

[0147] In addition, Figure 11 shows the marker expression of the sphere immediately before freezing in the same lot as the sphere shown in Figure 6C, and Table 5 shows the results of measuring the marker expression in multiple lots.

[Table 5]

Lot	1	2	3	4	Average	SD
SOX1	0.0	0.1	1.1	0.0	0.3	0.5
PAX6	0.0	0.0	0.0	0.5	0.1	0.3
KI67	20.0	20.1	7.7	16.9	16.2	5.8
NURR1	27.8	38.9	8.5	40.8	29.0	14.8
TH	13.3	12.4	9.6	12.0	11.8	1.6

[0148] Also, the expression rates of FOXA2 and LMX1A were compared (Table 6).

[Table 6]

LOT	1	2	3	4	Average	SD
LMX1A	86.5	87.8	64.8	92.6	82.9	12.4
FOXA2	80.9	88.1	70.3	90.8	82.5	9.1
LMX1A/FOXA2	78.5	82.6	57.7	85.5	76.1	12.6

[0149] A global PCA analysis using the microarray data was also performed to overview the temporal changes during maturation after the cryopreservation/thawing process. A total of 21,882 probe sets containing significant signals were used for the analysis. The PC1 and PC2 contributions were 48.0% and 15.7%, respectively. PC score plots were clustered into three main groups (day 0 (iPSC), day 12, and after day 28) (Figure 6H). The PCA plots for both unfrozen samples and cryopreserved samples shifted in the positive direction on the PC1 and PC2 axes, regardless of cryopreservation on day 28 and after day 28. Only 206 (0.88%) out of 23349 probe sets showed an increase or decrease more than 2-fold between the unfrozen samples and the cryopreserved samples in the same differentiation batch. In contrast, 222 (0.95%) out of 23275 probe sets showed an increase or decrease more than 2-fold between the unfrozen samples in different batches (Figures 6I, J). This result strongly indicates that the cryopreservation process has not affected the cell properties of the dopamine-producing neuron progenitor cells.

[0150] To confirm functional maturation of the iPSC-derived dopamine-producing neuron progenitor spheres, an electrophysiological analysis and a dopamine release measurement were performed. When the cryopreserved spheres were dissociated and cultured on the plate for further maturation, most of the cells gave rise to $TH^+/TUBB3^+$ dopamine-producing nerves on day 28 + 21 (Figure 6K). At this time point, continuous action potentials from the mature dopamine-producing nerves were detected by the whole-cell patch clamp technique, in which current is kept constant (Figure 6L). Dopamine secretion in the unfrozen spheres on day 56 and in the cryopreserved spheres on day 28 + 28 was also detected by LC/MS/MS. The amount of released dopamine was comparable to that of the unfrozen sphere (Figure 6M). Therefore, it was found that cell number and function of the cryopreserved iPSC-derived dopamine-producing neuron progenitor spheres are maintained.

(4) Engraftment of cryopreserved iPSC-derived dopamine-producing neuron progenitor spheres, and behavior of 6-OHDA lesioned rats

[0151] To confirm that the cryopreserved iPSC-derived dopamine-producing neuron progenitor cells could survive after transplantation, the unfrozen sphere and the cryopreserved sphere were transplanted into several types of rat corpus striatum and observed for a short period, followed by determination of a graft survival rate and number of survived cells. The results are shown in Table 7. There was no difference in number of rats with surviving grafts between both of the groups. When the spheres cryopreserved with a proton freezer and BBK were transplanted, the percentage of total surviving cells (HNA^+) was 42 ± 20% of that of the unfrozen cells.

Table 7. Results of a short-term validation experiment evaluating the graft survival rate of the cryopreserved spheres.

[Table 7]

	Animal		Duration	Number of animals with cells		Average number of survived cells	
	strain	Model		Unfrozen	Frozen	Unfrozen	Frozen
1383D6	Nude	Nomal	1 month	3/3	3/3	25555	4554

(continued)

	Animal		Duration	Number of animals with cells		Average number of survived cells	
	strain	Model		Unfrozen	Frozen	Unfrozen	Frozen
1231A3	Nude	Nomal	1 month	2/3	2/3	13589	8330
FF-I01	SD	6-OHDA lesioned	3 months	2/2	1/1	31781	18326
FF-I01	Nude	6-OHDA lesioned	2 weeks	3/3	3/3	31685	11520

[0152] The results of a short-term validation experiment with transplantation indicate that more than twice cryopreserved cells should be transplanted as compared to the unfrozen cells in order to achieve comparable number of survived cells *in vivo*. Therefore, to evaluate number of survived cryopreserved cells and their pharmacological effect, the unfrozen spheres (4×10^5 cells) and the cryopreserved spheres (8×10^5 cells) were transplanted into 6-OHDA lesioned PD disease model rats to measure their methamphetamine-induced rotational movements. Twenty four weeks after the transplantation, abnormal rotations were reduced in both of the groups (Figure 8A) . Immunofluorescent staining at 24 weeks confirmed cell engraftment in all rats that showed behavioral recovery. 55996 $\pm$ 3603 HNA^+ cells were survived in the graft derived from the unfrozen spheres, and 36486 $\pm$ 3578 HNA^+ cells were survived in the graft derived from the cryopreserved spheres (Figures 8B, C). For number of survived HNA^+ cells, no significant difference was found between both of the groups. Both of the groups had TH^+ cells survived and showed normal neurite extension (Figures 8D to G). There was no difference in number of TH^+ cells between the graft derived from the unfrozen spheres and the graft derived from the cryopreserved spheres (3603 $\pm$ 1576 cells/graft and 3578 $\pm$ 1490 cells/graft) (Figure 8H). TH^+ cells survived in the grafts derived from the unfrozen spheres and the cryopreserved spheres were 1.1 $\pm$ 0.4% and 0.5 $\pm$ 0.2% of injected cells, respectively. This result means that about 50% of TH^+ nerves survived in the graft derived from the cryopreserved spheres compared to that of the unfrozen spheres. Further, the most of the surviving cells in both of the groups were $FOXA2^+$ dopamine-producing neuron progenitor cells: 71 $\pm$ 6% for the unfrozen spheres and 76 $\pm$ 4% for the cryopreserved spheres (Figures 8I, J). In contrast, $KI67^+$ proliferating cells, which may cause the risk of abnormal proliferation, were quite few in both of the groups (Figures 8I, K). Therefore, it was found that the cryopreserved iPSC-derived dopamine-producing neuron progenitor spheres are engrafted and improve the behavior of 6-OHDA lesioned rats.

(5) Expression of dopamine-producing neuron progenitor cell markers

[0153] To confirm that the thawed cells have the ability to mature into dopamine-producing neurons similar to the unfrozen cells, protein markers and gene expression were examined over time until 7 days after thawing. As a result of immunocytochemistry, it was found that the cryopreserved spheres continued to express almost the same level of FOXA2 as compared to the unfrozen spheres (Figure 7A). NURR1+ cells increase from day 28 to day 35 in unfrozen cells. The cells after thawing showed the increase of NURR1+ cells at 7 days after thawing, which was slightly later than the unfrozen cells (Figure 7B). Further, a qPCR analysis showed that the expression level of TH was increased in the unfrozen cells with culturing period until day 35. It was confirmed that the expression of TH remained constant in the cells from day 0 to day 3 after thawing, and was increased on day 7 (Figure 7C). Therefore, it was found that the cryopreserved iPSC-derived dopamine-producing neuron progenitor spheres maintain the ability to mature into dopamine-producing neurons.

[0154] In addition, Table 8 shows the results of comparing unfrozen cells cultured up to day 35 with the cells frozen on day 28 and cultured for 7 days after thawing in multiple lots.

[Table 8]

LOT		1	2	3	4	Average	SD
D35	SOX1	0.5	0.0	0.0	0.3	0.2	0.2
	PAX6	0.0	0.0	0.4	0.2	0.1	0.2
	KI67	10.4	6.1	4.3	8.4	7.3	2.7
	FOXA2	82.9	90.4	83.0	88.8	86.3	3.9
	NURR1	36.8	51.3	9.5	28.5	31.5	17.5
	TH	27.0	18.4	18.8	15.8	20.0	4.9

(continued)

LOT		1	2	3	4	Average	SD
D28+7	SOX1	0.0	0.4	0.0	0.0	0.1	0.2
	PAX6	0.0	0.0	0.5	0.0	0.1	0.3
	KI67	13.3	7.5	16.9	4.6	10.6	5.5
	FOXA2	83.2	88.8	81.1	82.0	83.8	3.5
	NURR1	44.7	41.0	23.9	46.1	38.9	10.2
	TH	15.9	9.0	23.3	20.2	17.1	6.2

(6) Comparison of thawing conditions (reference)

[0155] The thawing conditions for cryopreserved spheres frozen with a proton freezer using BBK as a cryopreservation solution were examined. The tubes stored in the vapor phase of liquid nitrogen were taken out and thawed under three conditions of: (i) room temperature; (ii) a 37°C water bath; and (iii) melting at about 3°C/min (actual value) using a programmed freezer. The times required for thawing were (i) 17 minutes, (ii) 2 minutes, and (iii) about 25 minutes, respectively. The survival rate (recovery of viable cells) and neurite extension activity of the spheres after thawing were measured by the methods described above. As shown in Figure 9, thawing in the 37°C water bath gave the best result.

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Claims

1. A method for freezing a cell aggregate including neural cells and having a three-dimensional structure, which comprises following steps (1) and (2):

   (1) contacting the cell aggregate including neural cells and having the three-dimensional structure with a preservation solution at 0°C to 30°C prior to freezing to prepare a preservation solution-soaked cell aggregate; and (2) cooling the preservation solution-soaked cell aggregate obtained in step (1) from a temperature at least about 5°C higher than the freezing point of the preservation solution to a temperature about 5°C lower than the freezing point at an average cooling speed of 2 to 7 °C/min to freeze the cell aggregate.

2. The method according to claim 1, wherein the average cooling speed in step (2) is 3 to 7 °C/min.

3. The method according to claim 1 or 2, wherein the cell aggregate is contacted with the preservation solution for 15 minutes to 90 minutes, preferably 15 minutes to 60 minutes in step (1).

4. The method according to any one of claims 1 to 3, wherein the preservation solution has the freezing point of from -1°C to -10°C.

5. The method according to any one of claims 1 to 4, wherein the preservation solution is an aqueous solution comprising 7% to 12% of dimethyl sulfoxide and/or propylene glycol, and step (2) is a step of cooling from 0 ± 5°C to -30 ± 5°C at an average cooling speed of 2 to 5 °C/min.

6. The method according to any one of claims 1 to 5, wherein the average cooling speed in step (2) is 3 to 5 °C/min.

7. The method according to any one of claims 1 to 6, wherein the method further comprises a following step (3):
   (3) cooling the frozen cell aggregate obtained in step (2) to -50°C or lower.

8. The method according to any one of claims 1 to 7, wherein the cell aggregate including neural cells is a cell aggregate including neural cells derived from pluripotent stem cells.

9. The method according to any one of claims 1 to 8, wherein the cell aggregate including neural cells comprises cells that are positive for at least one of FOXA2, TH, and NURR1.

10. The method according to claim 9, wherein the cell aggregate including neural cells comprises cells positive for FOXA2 and LMX1A.

11. The method according to claim 9, wherein the cell aggregate including neural cells comprises cells positive for FOXA2, TH, and NURR1.

12. The method according to any one of claims 1 to 8, wherein the cell aggregate including neural cells comprises cells positive for FOXA2 and LMX1A in an amount of 40% or more of the total cells and cells positive for TH and NURR1 in an amount of 40% or less of the total cells.

13. The method according to any one of claims 1 to 12, wherein the cell aggregate including neural cells comprises a dopamine-producing neuron progenitor cell and/or a dopamine-producing neuron.

14. The method according to any one of claims 1 to 13, wherein the cell aggregate includes 500 to 150000 cells.

15. The method according to any one of claims 1 to 14, wherein number of cells contained in the preservation solution is 80000 to 5000000 cells/mL, and the cell aggregate has an equivalent spherical diameter of 150 to 1000 μm.

16. The method according to any one of claims 1 to 15, wherein the cell aggregate and the preservation solution have

volume of 0.25 mL to 2 mL.

17. The method according to any one of claims 1 to 16, wherein the cell aggregate and the preservation solution are packed in a 0.5 mL to 15 mL container.

18. A method for preserving a cell aggregate including neural cells and having a three-dimensional structure for a long-term, wherein the method comprises holding the frozen cell aggregate obtained by the method according to any one of claims 1 to 17 at or below -80°C.

19. The method according to any one of claims 1 to 18, wherein the obtained frozen cell aggregate can be used without recovery culture after thawing.

20. A composition for transplantation, wherein the composition comprises, as an active ingredient, the cell aggregate frozen or preserved for a long-term by the method according to any one of claims 1 to 19.

21. A frozen composition for transplantation, wherein the composition comprises:

a cell aggregate including 60% or more of dopamine-producing neuron progenitor cells and dopamine-producing neurons derived from a pluripotent stem cell, having an equivalent spherical diameter of 150 μm to 1000 μm and including 500 to 150000 cells; and
a cryopreservation solution comprising 7% to 12% of dimethyl sulfoxide or propylene glycol and having the freezing point of -1°C to -10°C, and
wherein the frozen cell aggregate has following properties:

(1) about 60% or more of the total cells are viable after thawing;
(2) the cells after thawing have a neurite extension activity of 50% or more of that before freezing; and
(3) the rate of the FOXA2-positive cells, LMX1A-positive cells, NURR1-positive cells, and TH-positive cells in the cells viable after thawing are changed within ± 10% from those in the cells before freezing.

22. The composition for transplantation according to claim 20 or 21, wherein number of cells in the composition is 80000 to 5000000 cells/mL, and the composition comprises cells positive for FOXA2 and LMX1A in an amount of 40% or more of the total cells and cells positive for TH and NURR1 in an amount of 40% or less of the total cells.

23. The composition for transplantation according to any one of claims 20 to 22, wherein the cell aggregate has an equivalent spherical diameter of 150 to 1000 μm.

24. The composition for transplantation according to any one of claims 20 to 23, wherein the composition can be used without recovery culture after thawing.

25. The composition for transplantation according to any one of claims 20 to 24, wherein the composition comprises 8 to 192 cell aggregates/mL, the cell aggregate has an equivalent spherical diameter of 150 μm to 1000 μm, and number of cells per container is 80000 to 2400000.

26. The composition for transplantation according to any one of claims 20 to 25, wherein the cell aggregate and the preservation solution have volume of 0.25 mL to 2 mL.

27. The composition for transplantation according to any one of claims 20 to 26, wherein the composition is packed in a 0.5 mL to 15 mL container.

28. A method for producing a composition for transplantation comprising a dopamine-producing neuron progenitor cell as an active ingredient, which comprises:
freezing a cell aggregate by the method according to any one of claims 1 to 17, wherein number of cells in the composition is 80000 to 5000000 cells/mL, the cell aggregate comprises cells positive for FOXA2 and LMX1A in an amount of 40% or more of the total cells and cells positive for TH and NURR1 in an amount of 40% or less of the total cells, and the cell aggregate has an equivalent spherical diameter of 150 μm to 1000 μm.

29. The method for producing the composition for transplantation according to claim 28 containing an aggregate of neural cells as an active ingredient, wherein the composition comprises a population of cell aggregates which have

an equivalent spherical diameter of 150 to 1000 μm, number of cells per container is 80000 to 2400000, the cell aggregate and the preservation solution have volume of 0.25 mL to 2 mL, and the composition is packed in a 0.5 mL to 15 mL container.

30. A method for treating a disease requiring regeneration of a dopamine-producing nerve, which comprises following steps of:

(1) thawing the composition for transplantation according to any one of claims 20 to 27 at 30°C to 40°C, preferably at 37°C ± 3°C; and
(2) transplanting the composition for transplantation obtained in (1) into a corpus striatum region of a patient.

31. The method according to claim 30, wherein the cryopreservation solution is replaced with a dosing vehicle without culture after thawing to perform step (2).

FIG. 1

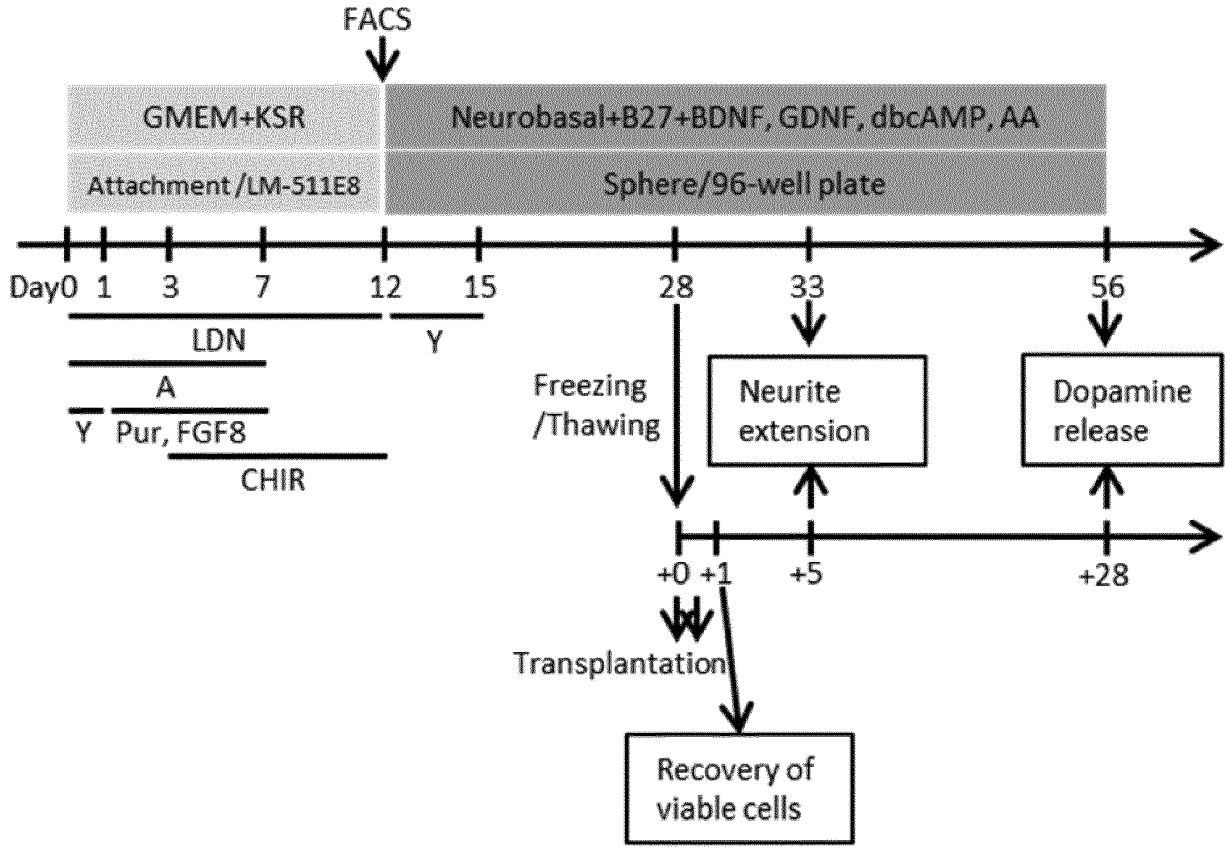


FIG. 2A

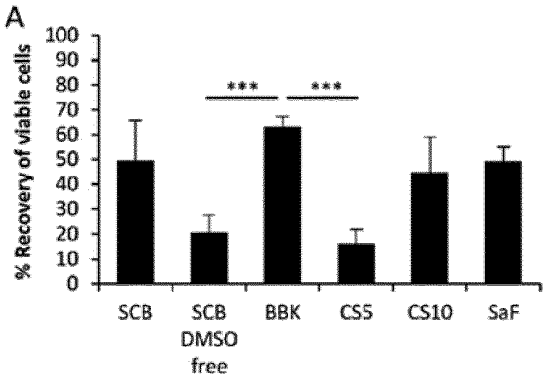


FIG. 2B

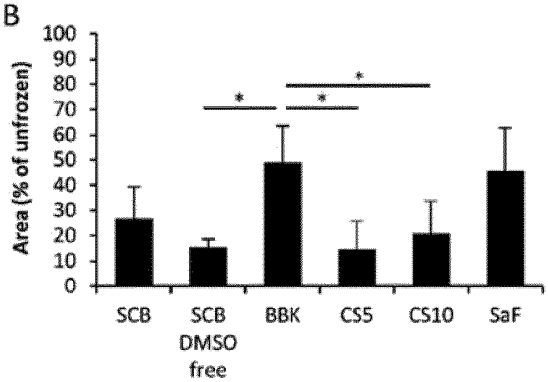


FIG. 2C

C

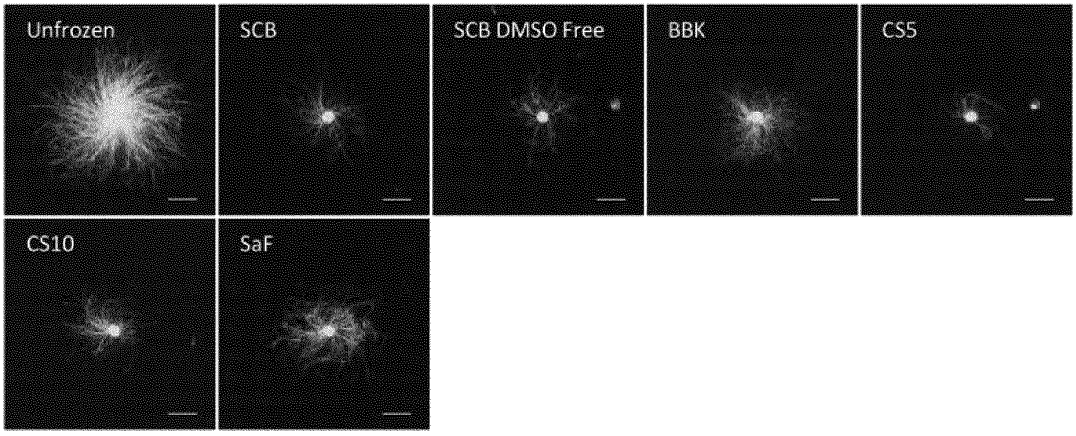

Unfrozen
SCB
SCB DMSO Free
BBK
CS5
CS10
SaF

FIG. 3

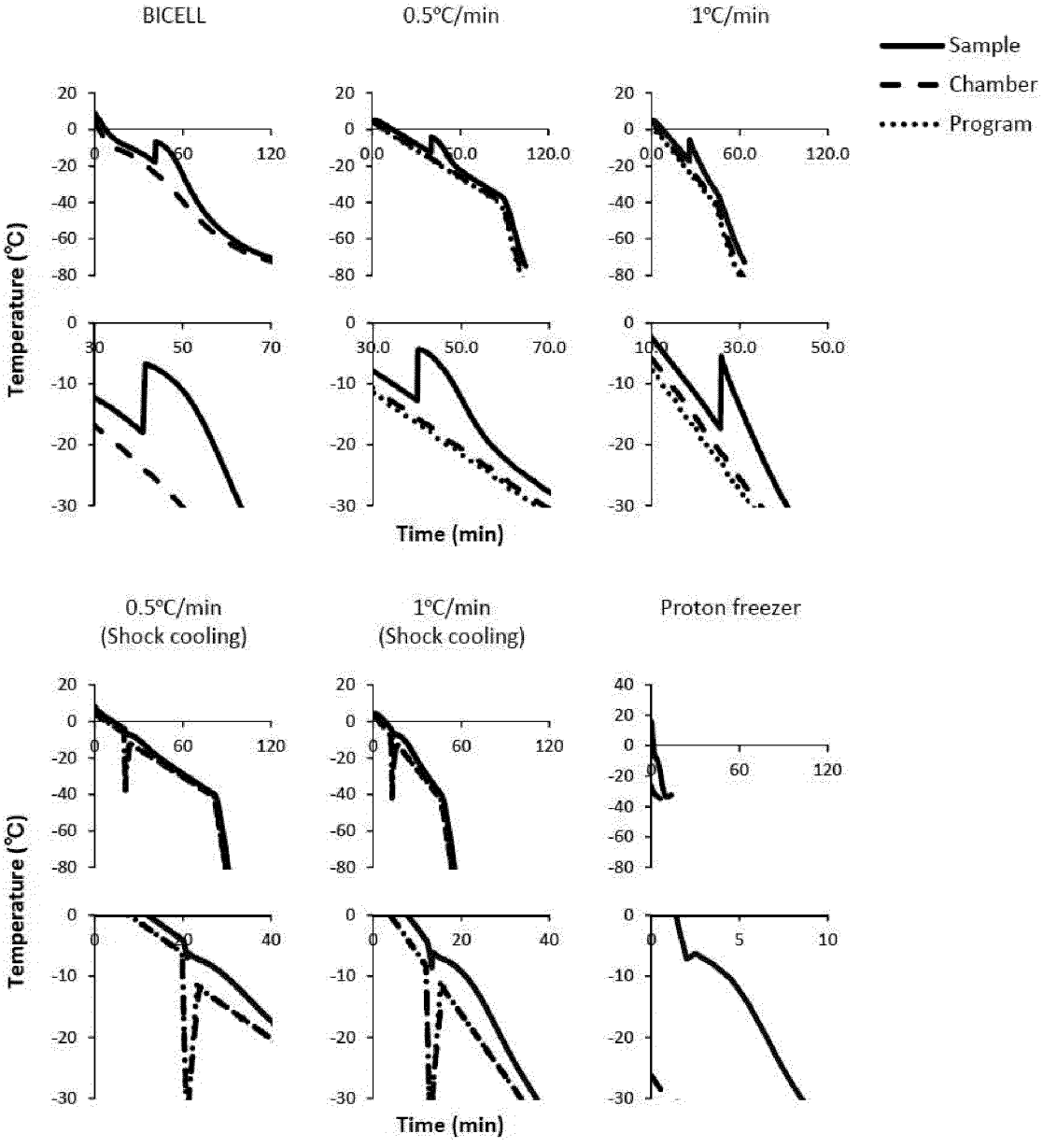

BICELL
0.5°C/min
1°C/min
Sample
Chamber
Program
Temperature (°C)
Time (min)
0.5°C/min
(Shock cooling)
1°C/min
(Shock cooling)
Proton freezer
Temperature (°C)
Time (min)

FIG. 4A

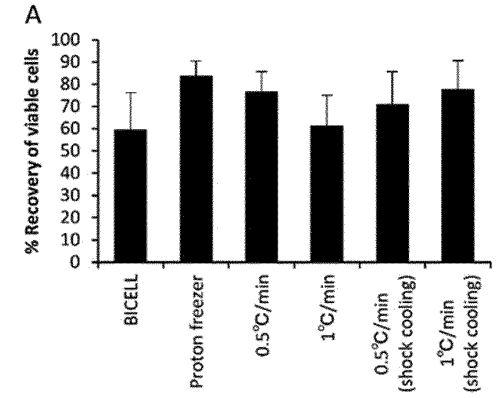


FIG. 4B

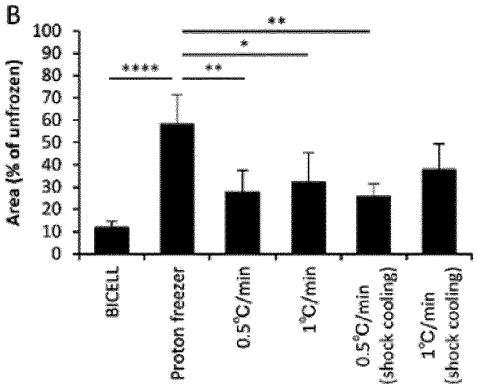


FIG. 5A

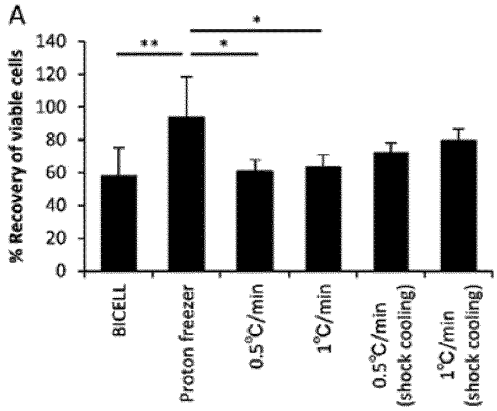


FIG. 5B

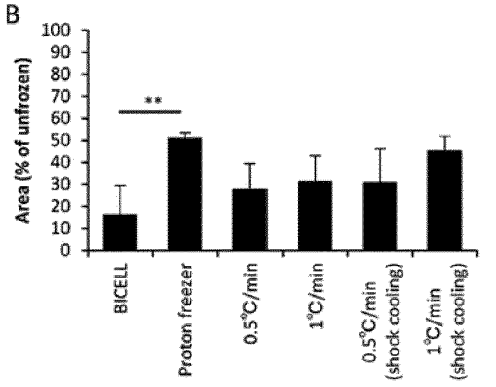


FIG. 6A

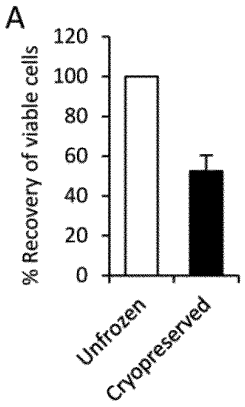


FIG. 6B

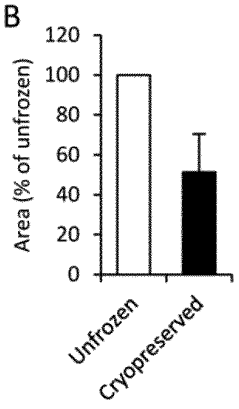


FIG. 6C

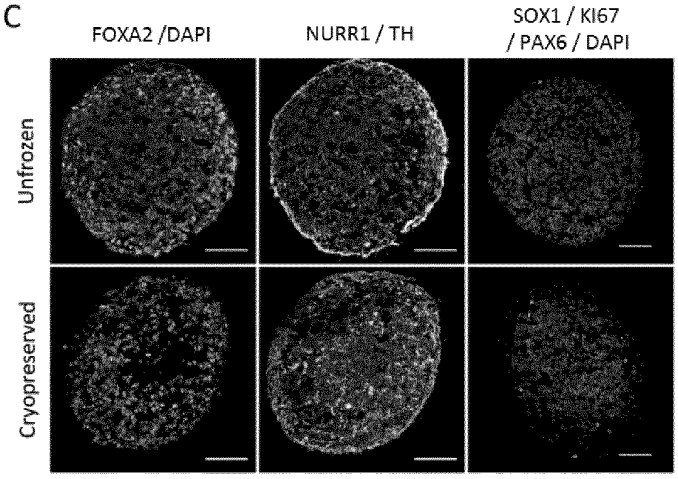


FIG. 6D

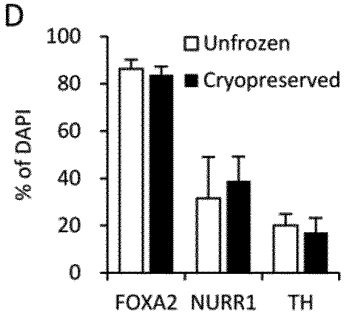


FIG. 6E

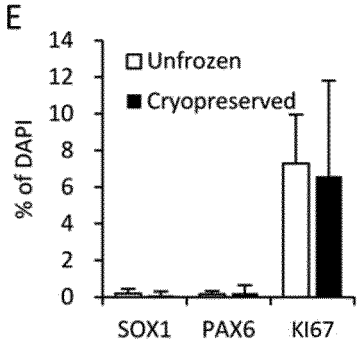


FIG. 6F

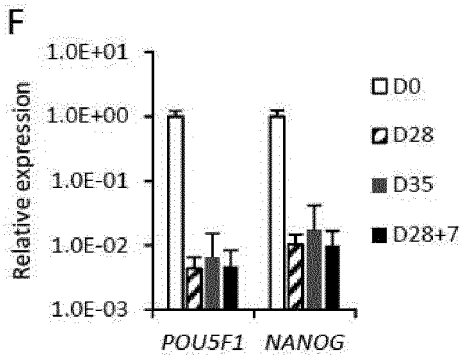


FIG. 6G

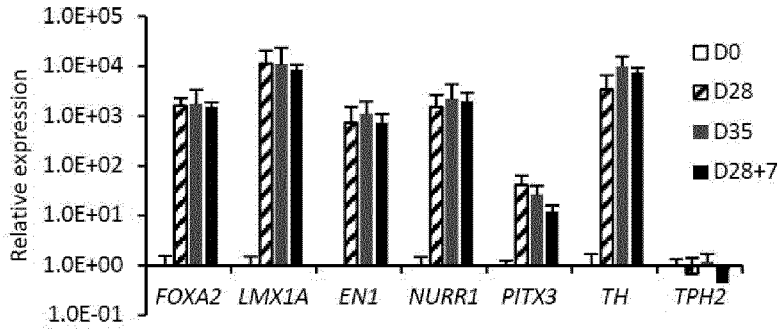


FIG. 6H

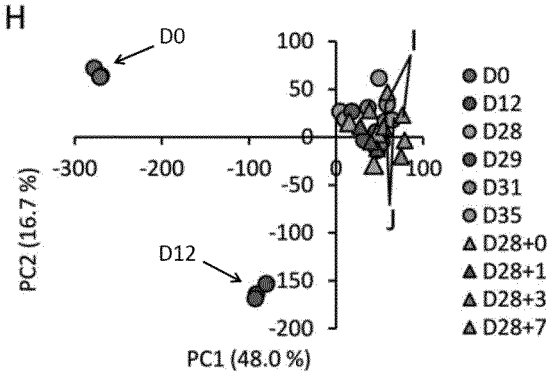


FIG. 6I

I

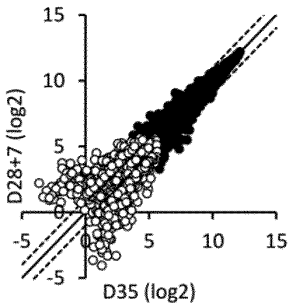


FIG. 6J

J

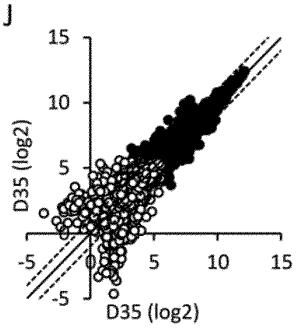


FIG. 6K

K

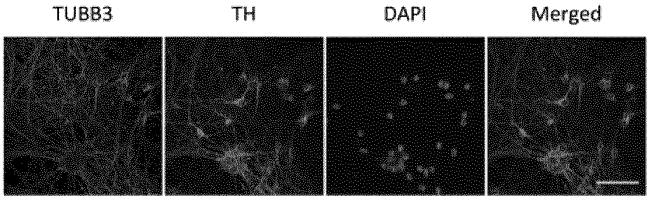


FIG. 6L

L

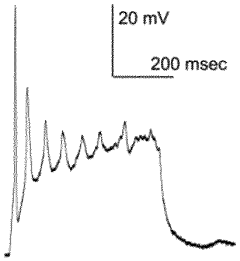


FIG. 6M

M

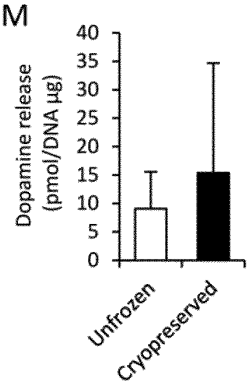


FIG. 7A

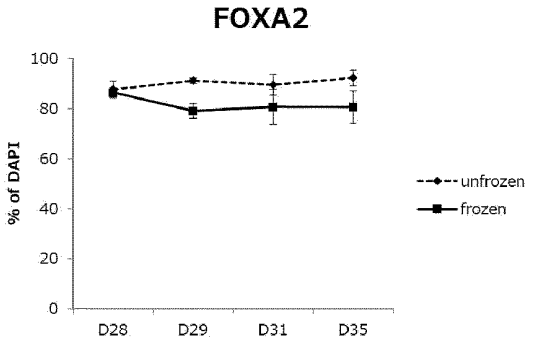


FIG. 7B

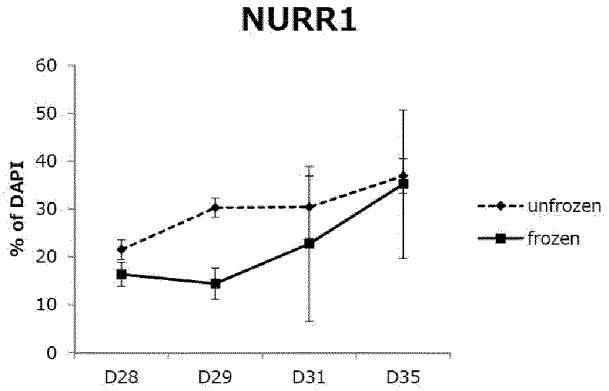


FIG. 7C

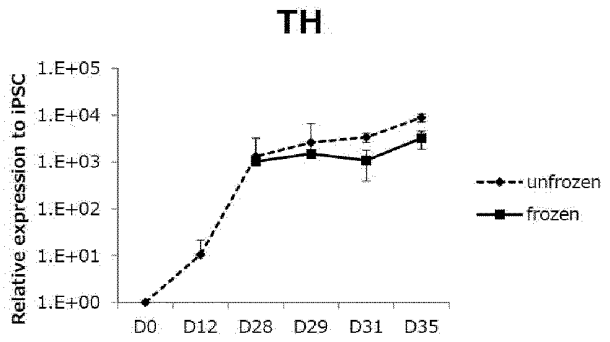


FIG. 8A

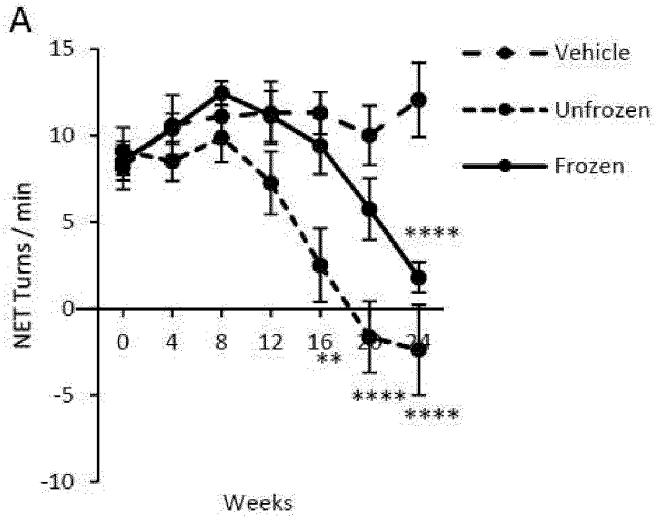


FIG. 8B

B

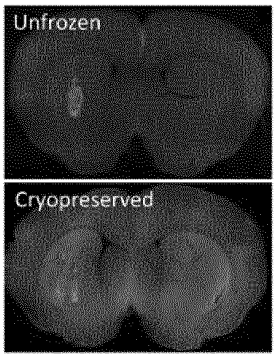


FIG. 8C

C

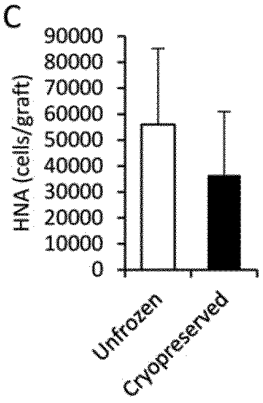


FIG. 8D

D

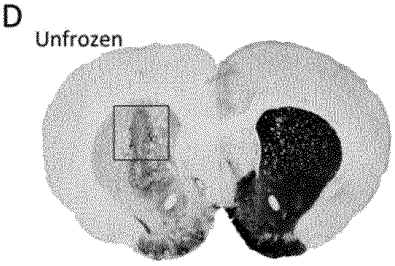


FIG. 8E

E

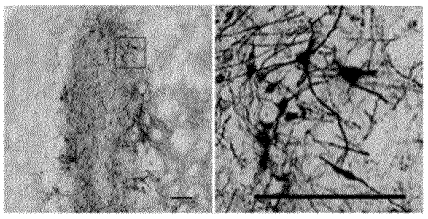


FIG. 8F

F

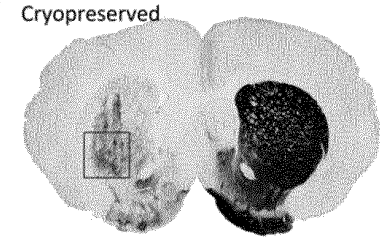


FIG. 8G

G

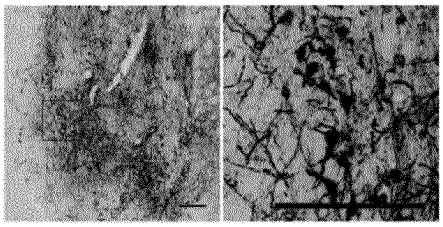


FIG. 8H

H

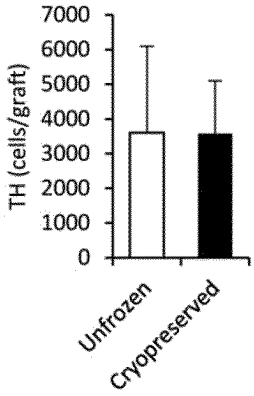


FIG. 8I

I

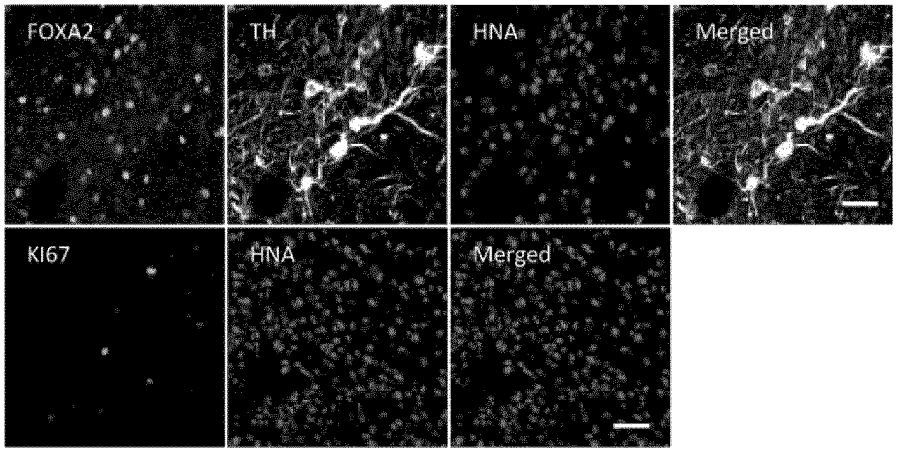


FIG. 8J

J

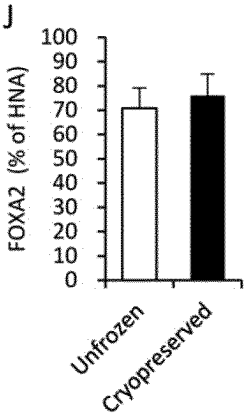


FIG. 8K

K

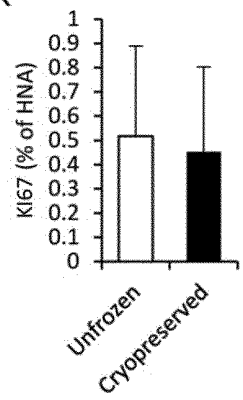


FIG. 9

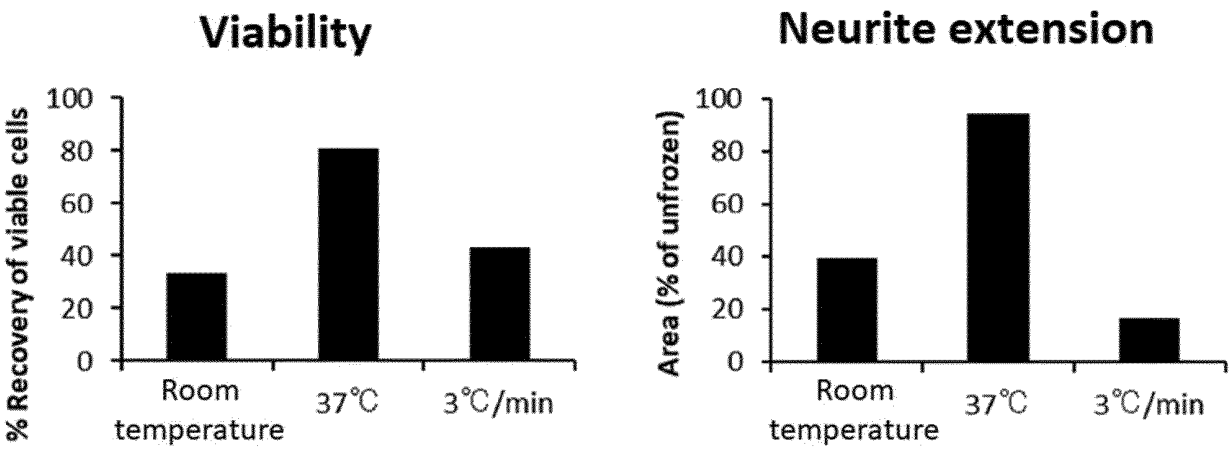


FIG. 10A

A

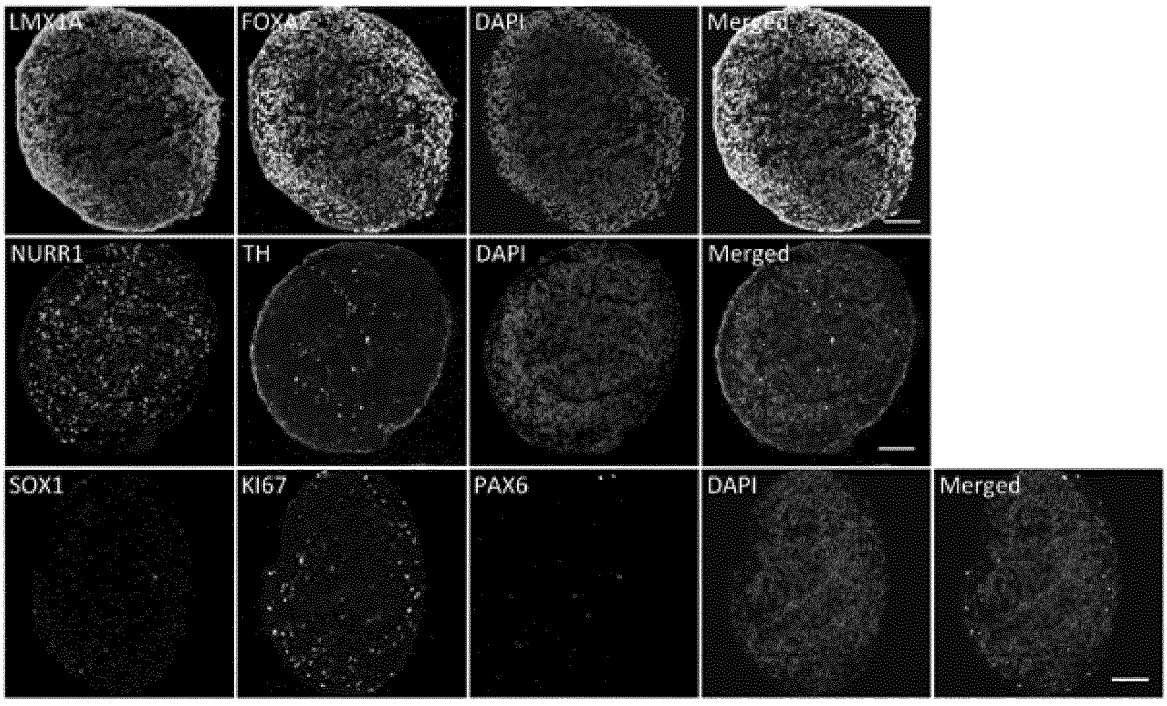


FIG. 10B

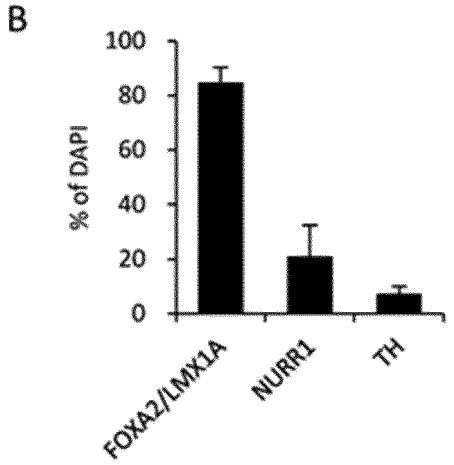


FIG. 10C

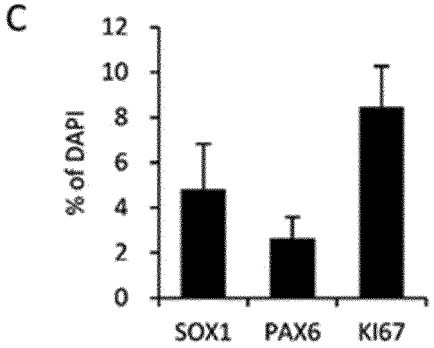
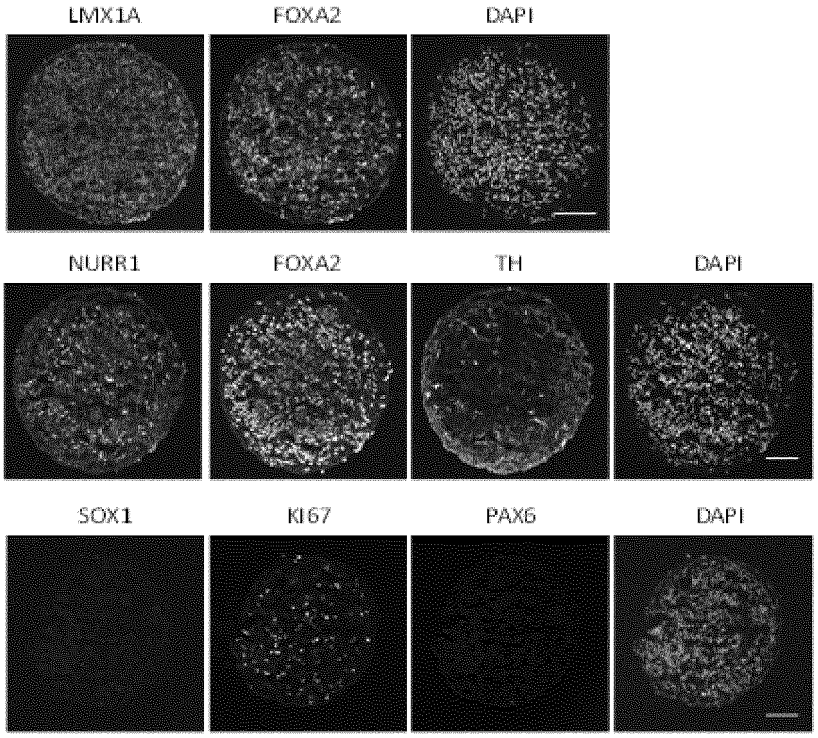


FIG. 11



INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2020/043275

A. CLASSIFICATION OF SUBJECT MATTER
C12N 5/10(2006.01)i; A61K 35/545(2015.01)i; A61P 25/16(2006.01)i; A61P 43/00(2006.01)i; C12N 1/04(2006.01)i; C12N 5/0793(2010.01)i
FI: C12N5/10; A61K35/545; A61P25/16; A61P43/00 107; A61P43/00 111; C12N5/0793; C12N1/04 ZNA
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N5/10; A61K35/545; A61P25/16; A61P43/00; C12N1/04; C12N5/0793

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Published examined utility model applications of Japan	1922-1996
Published unexamined utility model applications of Japan	1971-2021
Registered utility model specifications of Japan	1996-2021
Published registered utility model applications of Japan	1994-2021

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAplus/MEDLINE/EMBASE/BIOSIS/WPIDS (STN) JSTPlus/JMEDPlus/JST7580 (JDreamIII)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP 2013-110988 A (SUMITOMO CHEMICAL CO., LTD.) 10 June 2013 (2013-06-10) claims	1-31
A	JP 2008-501320 A (ES CELL INTERNATIONAL PTE LTD.) 24 January 2008 (2008-01-24) claims	1-31
A	JP 7-500597 A (CELLPRO, INCORPORATED) 19 January 1995 (1995-01-19) claims	1-31

☐ Further documents are listed in the continuation of Box C.
☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 January 2021 (05.01.2021)	19 January 2021 (19.01.2021)

Name and mailing address of the ISA/	Authorized officer
Japan Patent Office 3-4-3, Kasumigaseki, Chiyoda-ku, Tokyo 100-8915, Japan	Telephone No.

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/JP2020/043275

Patent Documents referred in the Report	Publication Date	Patent Family	Publication Date
JP 2013-110988 A	10 Jun. 2013	WO 2013/077424 A1 claims US 2014/0342346 A1 EP 2784154 A1	
JP 2008-501320 A	24 Jan. 2008	WO 2005/118785 A1 claims US 2008/0057040 A1 EP 1765986 A1	
JP 7-500597 A	19 Jan. 1995	WO 1993/007745 A1 claims EP 609379 A1	

Form PCT/ISA/210 (patent family annex) (January 2015)

REFERENCES CITED IN THE DESCRIPTION

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- JP 2015521469 A **[0008]**
- JP 2008501320 A **[0008]**
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- JP 2013110988 A **[0008]**
- WO 2015034012 A **[0033]**
- WO 2009148170 A **[0033]**
- WO 2013065763 A **[0033]**
- WO 2016013669 A **[0033]**
- WO 2017126551 A **[0033]**
- WO 2016039317 A **[0033]**
- WO 2015076388 A **[0033]**
- WO 9622362 A **[0036]**
- WO 02101057 A **[0036]**
- US 5843780 A **[0036]**
- US 6200806 B **[0036]**
- US 6280718 B **[0036]**

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- **PICCINI et al.** *Nature Neuroscience,* 1999, vol. 2 (12), 1137-1140 **[0009]**
- **DOI et al.** *Stem Cell Reports,* 2014, vol. 2 (3), 337-350 **[0009]**
- **SUNDBERG et al.** *Stem Cells,* 2013, vol. 31, 1548-1562 **[0009]**
- **NOLBRANT et al.** *Nature Protocols,* 2017, vol. 12 (9), 1962-1979 **[0009]**
- **REDMOND et al.** *Neurobiology of Disease,* 2008, vol. 29 (1), 103-116 **[0009]**
- **FRICKER et al.** *PLoS ONE,* 2012, vol. 7 (10), e47169 **[0009]**
- **FRODL et al.** *Brain Research,* 1994, vol. 647 (2), 286-298 **[0009]**
- **SAUTTER et al.** *Journal of Neuroscience Methods,* 1996, vol. 64 (2), 173-179 **[0009]**
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(54) **Title:** METHOD FOR FREEZING NEURAL CELLS

(54) 発明の名称： 神経系細胞の凍結方法

(57) **Abstract:** Provided is a method for freezing a cell aggregate that includes neural cells. Provided is a method for freezing a cell aggregate that includes neural cells having a three-dimensional structure, said method including the following step (1) and step (2): (1) a step in which, prior to freezing, the cell aggregate that includes the neural cells having the three-dimensional structure is brought into contact with a preservation liquid at 0 ° C to 30 ° C, and a preservation liquid-soaked cell aggregate is prepared; and (2) a step in which the preservation liquid-soaked cell aggregate that was obtained in step (1) is cooled at an average temperature reduction speed of 2-7 ° C/min, at least from a temperature that is approximately 5 ° C above the freezing point of the preservation liquid to a temperature that is approximately 5 ° C below said freezing point, and is thus made to freeze.

(57) 要約：神経系細胞を含む細胞凝集体を凍結する方法を提供する。以下の工程（１）及び工程（２）を含む、立体構造を有する神経系細胞を含む細胞凝集体を凍結する方法を提供する：(１)立体構造を有する神経系細胞を含む細胞凝集体を、凍結前に０℃以上３０℃以下で、保存液に接触させ、保存液に浸された細胞凝集体を調製する工程、及び；(２)工程（１）で得られる保存液に浸された細胞凝集体を、少なくとも、該保存液の凝固点よりも約５℃高い温度から、凝固点よりも約５℃低い温度まで、平均２～７℃／分の温度低下速度で冷却し、凍結させる工程。

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明　細　書

発明の名称 ： 神経系細胞の凍結方法

技術分野

[0001] 本願は、神経系細胞を含む細胞凝集体の凍結方法に関する。

背景技術

[0002] ドパミン産生（DA）神経の移植は、胎児中脳細胞を用いた臨床試験の報告等から、パーキンソン病（PD）の有望な治療方法になると考えられている（非特許文献１）。また、胚性幹細胞（ES細胞又はESC）や人工多能性幹細胞（iPS細胞またはiPSC）等の多能性幹細胞（PSC）をドパミン産生神経細胞もしくはその前駆細胞へ分化誘導する方法が報告されている。本発明者らのグループは既に、ヒト人工多能性幹細胞からドパミン産生神経細胞、又はドパミン産生神経前駆細胞等を作製する方法を報告している（非特許文献２）。他のグループからも、PSC由来ドパミン産生神経の作製が報告されている（非特許文献３及び４）。

[0003] 細胞を有効成分とする医薬品においては、最終生成物の凍結保存の達成は細胞療法を広めるために不可欠な要素である（特許文献１～４）。細胞生物学の研究とは異なり、臨床に使用される場合、凍結保存細胞は解凍後回復培養を行わず直ちに移植されることが望ましい。したがって、凍結細胞としては解凍後に生着能、機能・活性および細胞生存率が維持されていることが重要である。

[0004] 固形組織の移植ではドナーの血管および抗原提示細胞がそのまま存在することにより、細胞懸濁液の移植よりも強い免疫反応が誘発されることが示唆されている（非特許文献５）。一方、同系移植または免疫抑制剤によって免疫応答が抑制されれば、この問題は解消され、腹側中脳（VM）組織の移植は細胞懸濁液の移植よりも高いドパミン産生神経の生存率および行動回復を示す（非特許文献６）。また、細胞懸濁液を取得するための機械的解離プロセスおよび酵素的解離プロセスは細胞特性を変化させ、細胞損傷を引き起こし

得る。したがって、臨床用途においては移植される細胞を細胞懸濁液ではなく、細胞塊として投与することが望ましい。しかし、細胞塊は単一細胞よりも凍結保存が困難であるという問題がある。

[0005] 凍結保存したPSC由来ドパミン産生神経の単一細胞懸濁液をラット線条体に移植すると、生存TH^+細胞の割合は非凍結細胞と比較して約60%に減少する（非特許文献７）。一方、ヒトまたはラット腹側中脳（VM）組織を凍結保存したほとんどの研究例では、インビボでのドパミン産生神経の生存率は非凍結組織と比較して20%未満にまで減少している（非特許文献８～１０）。したがって、PSC由来ドパミン産生神経細胞塊の生存を維持することが可能な凍結方法の開発が必要とされている。

[0006] 一般的に、２つの細胞凍結保存法が存在する（非特許文献１１～１３）。このうち緩慢法は低濃度の凍結保護物質（CPA）（10%ジメチルスルホオキシド（DMSO）など）と共に細胞を約1℃／分で凍結させる方法である（特許文献５、非特許文献１４、１５）。一方ガラス化法は、高濃度の凍結保護物質を添加した後、直ちに細胞を液体窒素中に移行させる高速冷却法である（特許文献６、非特許文献１６）。ガラス化法は厳密な時間制御を必要とするため、臨床用細胞製造への適用は技術的に困難である（非特許文献１７）。

[0007] 一方、緩慢法では、氷形成がまず細胞外空間において開始し、細胞外液の濃縮をもたらす。その結果、細胞膜を隔てた浸透圧勾配によって細胞から水分が引き抜かれる。この細胞の脱水によって細胞内氷形成が回避される。しかしながら、過度に細胞が脱水された場合、濃縮された細胞内液および凍結保存液中のCPAにより細胞は傷害される。氷形成及び細胞脱水の正確な制御が必要であることから、細胞塊の臨床用凍結保存法は確立されていなかった。

先行技術文献

特許文献

[0008] 特許文献1：特開２０１７－１０４０６１号公報
特許文献2：ＷＯ２０１７／１５９８６２号明細書
特許文献3：特表２０１５－５２１４６９号公報

特許文献4：特表２００８－５０１３２０号公報

特許文献5：特開２０１１－１０３８８５号公報

特許文献6：特開２０１３－１１０９８８号公報

非特許文献

[0009] 非特許文献1：Piccini et al; Nature Neuroscience, 2(12), 1137-1140, 1999

非特許文献2：Doi et al., Stem Cell Reports, 2(3), 337-350, 2014

非特許文献3：Sundberg et al., Stem Cells 31, 1548-1562, 2013

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非特許文献5：Redmond et al., Neurobiology of Disease, 29(1), 103-116, 2008

非特許文献6：Fricker et al., PLoS ONE, 7(10), e47169, 2012

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非特許文献10：Sautter et al., Experimental Neurology, 164(1), 121-129, 2000

非特許文献11：Chong et al., Stem Cells, 27(1), 29-39, 2009

非特許文献12：Smith et al., Fertility and Sterility, 94(6), 2088-2095, 2010

非特許文献13：Jang et al., Integrative Medicine Research, 6(1), 12-18, 2017

非特許文献14：Schwartz et al., Journal of Neuroscience Research, 74(6), 838-851, 2003

非特許文献15：Woods et al., Cryobiology, 59(2), 150-157, 2009

非特許文献16：Fahy and Wowk., Methods in Molecular Biology (Methods and Protocols), vol 1257. Springer, New York, NY, 2015

非特許文献17：Nagano et al., Biomedical Research, 28(3), 153-160, 2007

発明の概要

発明が解決しようとする課題

[0010] 本願は、神経系細胞を含む細胞凝集体の凍結方法を提供することを目的とする。

課題を解決するための手段

[0011] 本発明者らは鋭意検討を行った結果、神経系細胞を含む細胞凝集体の凍結方法を見出し、本発明を完成するに至った。すなわち、本発明は、以下：

〔１〕以下の工程（１）及び工程（２）を含む、立体構造を有する神経系細胞を含む細胞凝集体を凍結する方法：

(１)立体構造を有する神経系細胞を含む細胞凝集体を、凍結前に０℃以上３０℃以下で、保存液に接触させ、保存液に浸された細胞凝集体を調製する工程、及び；

(２)工程（１）で得られる保存液に浸された細胞凝集体を、少なくとも、該保存液の凝固点よりも約５℃高い温度から、凝固点よりも約５℃低い温度まで、平均２～７℃／分の温度低下速度で冷却し、凍結させる工程。

〔２〕工程（２）における温度低下速度が、平均３～７℃／分である、前記〔１〕に記載の方法。

〔３〕工程（１）において、細胞凝集体を１５分間～９０分間、好ましくは１５分間～６０分間保存液に接触させる、前記〔１〕又は〔２〕に記載の方法。

〔４〕保存液の凝固点が、－１℃～－１０℃である、前記〔１〕～〔３〕のいずれか一項に記載の方法。

〔５〕保存液が、７%～１２%のジメチルスルホキシド及び/又はプロピレングリコールを含む水性液体であり、工程（２）が、０±５℃から－３０±５

℃まで、平均２～５℃／分の温度低下速度で冷却する工程である、前記〔１〕～〔４〕のいずれか一項に記載の方法。

〔６〕工程（２）における温度低下速度が、平均３～５℃／分である、前記〔１〕～〔５〕のいずれか一項に記載の方法。

〔７〕更に、以下の工程（３）を含む、前記〔１〕～〔６〕のいずれか一項に記載の方法：

（３）工程（２）で得られる凍結した細胞凝集体を、－５０℃以下に冷却する工程。

〔８〕神経系細胞を含む細胞凝集体が、多能性幹細胞由来の神経系細胞を含む細胞凝集体である、前記〔１〕～〔７〕のいずれか一項に記載の方法。

〔９〕神経系細胞を含む細胞凝集体が、FOXA2、TH及びNURR1の少なくとも１つが陽性の細胞を含む、前記〔１〕～〔８〕のいずれか一項に記載の方法。

〔１０〕神経系細胞を含む細胞凝集体が、FOXA2陽性かつLMX1A陽性の細胞を含む、前記〔９〕に記載の方法。

〔１１〕神経系細胞を含む細胞凝集体が、FOXA2陽性、TH陽性、かつNURR1陽性細胞を含む、前記〔９〕に記載の方法。

〔１２〕神経系細胞を含む細胞凝集体が、FOXA2陽性かつLMX1A陽性の細胞を全細胞数の４０％以上含み、TH陽性かつNURR1陽性の細胞を全細胞数の４０％以下含む、前記〔１〕～〔８〕のいずれか一項に記載の方法。

〔１３〕神経系細胞を含む細胞凝集体が、ドパミン産生神経前駆細胞及び／又はドパミン産生神経細胞を含む、前記〔１〕～〔１２〕のいずれか一項に記載の方法。

〔１４〕細胞凝集体が、５００個～１５００００個の細胞を含む、前記〔１〕～〔１３〕のいずれか一項に記載の方法。

〔１５〕保存液中に含まれる細胞数が８００００～５０００００００ｃｅｌｌｓ／ｍＬであり、細胞凝集体の円相当径が１５０～１０００μｍである、前記〔１〕～〔１４〕のいずれか一項に記載の方法。

〔１６〕細胞凝集体及び保存液の体積が０．２５ｍＬ～２ｍＬである、前記

〔１〕～〔１５〕のいずれか一項に記載の方法。

〔１７〕細胞凝集体及び保存液が、０．５ｍｌ～１５ｍｌの容器に充填されている、前記〔１〕～〔１６〕のいずれか一項に記載の方法。

〔１８〕前記〔１〕～〔１７〕のいずれかに記載の方法で得られる凍結した細胞凝集体を、－８０℃以下で保持することを含む、立体構造を有する神経系細胞を含む細胞凝集体の長期保存方法。

〔１９〕解凍後に回復のための培養を行うことを要しない、凍結された細胞凝集体を得ることを特徴とする、前記〔１〕～〔１８〕のいずれかに記載の方法。

〔２０〕前記〔１〕～〔１９〕のいずれか一項に記載の方法で凍結または長期保存された細胞凝集体を有効成分として含有する移植用組成物。

〔２１〕多能性幹細胞由来のドパミン産生神経前駆細胞及びドパミン産生神経細胞を６０％以上含む、円相当径１５０μｍ～１０００μｍであって、５００個～１５００００個の細胞を含む細胞凝集体、及び７％～１２％のジメチルスルホキシドもしくはプロピレングリコールを含む凝固点が－１℃～－１０℃の凍結保存液を含み、以下の性質：

（１）解凍後に全細胞数の約６０％以上が生存している、

（２）凍結前と比較して、５０％以上の神経突起伸長活性を有する、

（３）解凍後に生存している細胞におけるFOXA2、LMX1A、NURR1およびTHの陽性率の変化が±１０％以内である、を示す、凍結された移植用組成物。

〔２２〕細胞数が８００００～５０００００００ｃｅｌｌｓ／ｍＬであり、FOXA2陽性かつLMX1A陽性の細胞を全細胞数の４０％以上含み、TH陽性かつNURR1陽性の細胞を全細胞数の４０％以下含む、前記〔２０〕又は〔２１〕に記載の移植用組成物。

〔２３〕細胞凝集体の円相当径が１５０～１０００μｍである、前記〔２０〕～〔２２〕のいずれか一項に記載の移植用組成物。

〔２４〕解凍後に回復のための培養を行うことを要しないことを特徴とする、前記〔２０〕～〔２３〕のいずれか一項に記載の移植用組成物。

〔２５〕細胞凝集体を８～１９２個／ｍｌ含み、かつ、当該細胞凝集体の円相当径が１５０μｍ～１０００μｍであり、容器当たりの細胞数が８０００～２４０００００個である、前記〔２０〕～〔２４〕のいずれか一項に記載の移植用組成物。

〔２６〕細胞凝集体及び保存液の体積が０．２５ｍＬ～２ｍＬである、前記〔２０〕～〔２５〕のいずれか一項に記載の移植用組成物。

〔２７〕０．５ｍｌ～１５ｍｌの容器に充填されている、前記〔２０〕～〔２６〕のいずれか一項に記載の移植用組成物。

〔２８〕前記〔１〕～〔１７〕のいずれか一項に記載の方法で、細胞数が８００００～５０００００００ｃｅｌｌｓ／ｍＬであり、FOXA2陽性かつLMX1A陽性の細胞を全細胞数の４０％以上含み、TH陽性かつNURR1陽性の細胞を全細胞数の４０％以下含む、円相当径１５０μｍ～１０００μｍの細胞凝集体を凍結することを含む、ドパミン産生神経前駆細胞を有効成分として含有する移植用組成物の製造方法。

〔２９〕当該細胞凝集体の円相当径が１５０～１０００μｍである細胞凝集体の集団を含み、容器当たりの細胞数が８００００～２４０００００個であり、細胞凝集体及び保存液の体積が０．２５ｍＬ～２ｍＬである、０．５ｍｌ～１５ｍｌの容器に充填されている、神経系細胞の凝集体を有効成分として含有する前記〔２８〕に記載の移植用組成物の製造方法。

〔３０〕以下の工程を含む、ドパミン産生神経の再生を必要とする疾患の治療方法：

（１）前記〔２０〕～〔２７〕のいずれか一項に記載の移植用組成物を３０℃～４０℃、好ましくは３７℃±３℃において解凍する工程、

（２）（１）で得られた移植用組成物を、患者の線条体領域に移植する工程。

〔３１〕解凍後に、培養を行うことなく、凍結保存液を投与媒体に置換し、工程（２）を行うことを特徴とする、前記〔３０〕に記載の方法。

に関する。

発明の効果

[0012] 本願は、神経系細胞を含む細胞凝集体を凍結保存する方法を提供する。本願の方法によって凍結保存された神経系細胞は高い細胞生存率を示し、機能的性質を維持している。

図面の簡単な説明

[0013] [図1]iPSCからドパミン産生神経前駆細胞への分化誘導のプロトコルおよび評価実験のタイミングを示すスキーム。図中の略号は培地に添加される各成分を示す。LDN：LDN193189、A：A83-01、Y：Y-27632、Pur：パルモルファミン、CHIR：CHIR99021、AA：アスコルビン酸。

[0014] [図2A]iPSC由来ドパミン産生神経前駆細胞に対する凍結保存液の効果。表1に示される凍結保存液に15分間浸透させた後、0.5℃／分で凍結保存した、ソーティングされていない細胞の解凍後の生細胞回収率（n=4）。

[図2B]iPSC由来ドパミン産生神経前駆細胞に対する凍結保存液の効果。表1に示される凍結保存液に15分間浸透させた後、0.5℃／分で凍結保存した、ソーティングされていない細胞由来の細胞塊の神経突起伸長（n=4）。

[図2C]iPSC由来ドパミン産生神経前駆細胞に対する凍結保存液の効果。表1に示される凍結保存液に15分間浸透させた後、0.5℃／分で凍結保存した、ソーティングされていない細胞由来の細胞塊の神経突起を初期神経マーカー（PSA-NCAM）で染色した免疫染色像を示す。スケールバーは1mmを示す。

[0015] [図3]試料（直線）、冷凍チャンバー（破線）およびプログラム（点線）の時間－温度曲線。Bambanker hRMを試料として用いた。下の図は、上の図における潜熱放出による温度変化を拡大したものである。

[0016] [図4A]iPSC由来ドパミン産生神経前駆細胞に対する凍結プログラムの効果。Bambanker hRMに15分間浸透させた後、種々の凍結プログラムで凍結保存した、ソーティングされていない細胞の解凍後の生細胞回収率。

[図4B]iPSC由来ドパミン産生神経前駆細胞に対する凍結プログラムの効果。Bambanker hRMに15分間浸透させた後、種々の凍結プログラムで凍結保存した、ソーティングされていない細胞由来の細胞塊の神経突起伸長。

[0017] [図5A]iPSC由来ドパミン産生神経前駆細胞に対する、凍結保存液に長時間曝露した後の凍結の効果。種々の凍結プログラムにおいてBambanker hRMに60分間曝露した後に凍結保存された、ソーティングされていない細胞における解凍後の生細胞回収率。

[図5B]iPSC由来ドパミン産生神経前駆細胞に対する、凍結保存液に長時間曝露した後の凍結の効果。種々の凍結プログラムにおいてBambanker hRMに60分間曝露した後に凍結保存された、ソーティングされていない細胞由来の細胞塊の神経突起伸長。

[0018] [図6A]インビトロにおける凍結保存細胞塊の特徴。解凍後の生細胞回収率。

[図6B]インビトロにおける凍結保存細胞塊の特徴。細胞塊の神経突起伸長。

[図6C]インビトロにおける凍結保存細胞塊の特徴。35日目及び解凍後7日目における細胞塊の免疫染色。左図はFOXA2/DAPI、中央の図はNURR1/TH、右図はSOX1/KI67/PAX6/DAPIの免疫染色像を示す。スケールバーは100μmを示す。

[図6D]インビトロにおける凍結保存細胞塊の特徴。35日目及び解凍後7日目の全細胞に対するFOXA2$^+$細胞、NURR1$^+$細胞、TH$^+$細胞の割合。

[図6E]インビトロにおける凍結保存細胞塊の特徴。35日目及び解凍後7日目の全細胞に対するSOX1$^+$細胞、PAX6$^+$細胞およびKI67$^+$細胞の割合。

[図6F]インビトロにおける凍結保存細胞塊の特徴。定量RT-PCRで測定された、GAPDHに対する細胞塊の遺伝子発現。未分化細胞（0日目）の発現レベルを1に設定した。

[図6G]インビトロにおける凍結保存細胞塊の特徴。定量RT-PCRで測定された、GAPDHに対する細胞塊の遺伝子発現。未分化細胞（0日目）の発現レベルを1に設定した。

[図6H]インビトロにおける凍結保存細胞塊の特徴。マイクロアレイデータの主成分分析。非凍結細胞（円形）および凍結細胞（三角形）の遺伝子発現の時間変化を示す。

[図6I]インビトロにおける凍結保存細胞塊の特徴。35日目（X軸）または解凍後7日目（Y軸）における、同一ロットの非凍結細胞と凍結細胞のマイクロア

レイデータのスキャッタープロットを示す。黒丸はいずれか一方の検体でシグナル強度50以上を示す遺伝子、白丸は両検体でシグナル強度50以下の遺伝子を示す。

[図6J]インビトロにおける凍結保存細胞塊の特徴。35日目における、異なるロット間の非凍結細胞のマイクロアレイデータのスキャッタープロットを示す。黒丸はいずれか一方の検体でシグナル強度50以上を示す遺伝子、白丸は両検体でシグナル強度50以下の遺伝子を示す。

[図6K]インビトロにおける凍結保存細胞塊の特徴。28＋21日目の解凍後のiPSC由来ドパミン産生神経細胞におけるTUBB3、THおよびDAPIの免疫染色像。スケールバーは50μmを示す。

[図6L]インビトロにおける凍結保存細胞塊の特徴。28＋21日目における解凍後のiPSC由来ドパミン産生神経細胞の代表的な誘導活動電位。

[図6M]インビトロにおける凍結保存細胞塊の特徴。56日目または解凍後28＋28日目において高濃度カリウム刺激によって誘導されたドパミン放出量の結果。

[0019] [図7A]解凍後のドパミン産生神経前駆細胞マーカー発現の時間変化。28、29、31、35日目および解凍後0、1、3、7日目における全細胞に対するFOXA2＋細胞の割合。

[図7B]解凍後のドパミン産生神経前駆細胞マーカー発現の時間変化。28、29、31、35日目および解凍後0、1、3、7日目における全細胞に対するNURR1＋細胞の割合。

[図7C]解凍後のドパミン産生神経前駆細胞マーカー発現の時間変化。定量RT-PCRで測定された、GAPDHに対する細胞塊のTH遺伝子発現。未分化細胞（0日目）の発現レベルを1に設定した。

[0020] [図8A]凍結保存細胞塊の移植片生着率および機能。移植片を有するラットのメタンフェタミン誘発回転運動。データを平均値±SEM（n＝6～8）として示す。テューキーの多重比較検定を伴う二元配置分散分析を行い、有意水準を溶媒投与群に対する**p<0.01、****p<0.001によって示す。

[図8B]凍結保存細胞塊の移植片生着率および機能。非凍結細胞（上図）および凍結細胞（下図）由来の代表的な移植片におけるHNAの免疫染色像。

[図8C]凍結保存細胞塊の移植片生着率および機能。移植片における生存HNA^+細胞数。

[図8D]凍結保存細胞塊の移植片生着率および機能。非凍結細胞由来の代表的な移植片におけるTHのDAB染色像。

[図8E]凍結保存細胞塊の移植片生着率および機能。図８Ｄの枠内の拡大像。右側のパネルは左側のパネルの枠内の拡大画像である。スケールバーは200μmを示す。

[図8F]凍結保存細胞塊の移植片生着率および機能。凍結細胞由来の代表的な移植片におけるTHのDAB染色像。

[図8G]凍結保存細胞塊の移植片生着率および機能。図８Ｆの枠内の拡大像。右側のパネルは左側のパネルの枠内の拡大画像である。スケールバーは200μmを示す。

[図8H]凍結保存細胞塊の移植片生着率および機能。移植片における生存TH^+細胞数。

[図8I]凍結保存細胞塊の移植片生着率および機能。凍結細胞由来の移植片におけるFOXA2、TH、HNA（上側）、KI67、HNA（下側）の免疫染色像。スケールバーは50μmを示す。

[図8J]凍結保存細胞塊の移植片生着率および機能。HNA^+細胞に対する$FOXA2^+$細胞の割合。

[図8K]凍結保存細胞塊の移植片生着率および機能。HNA^+細胞に対する$KI67^+$細胞の割合。

[図9]凍結保存細胞塊を異なる条件で解凍した場合における、生存率及び神経突起伸長活性を示す。

[図10A]抗CORIN抗体でソーティングされていない細胞由来の細胞塊のマーカー発現。細胞塊におけるLMX1A、FOXA2、DAPI（上段）、NURR1、TH、DAPI（中段）、SOX1、KI67、PAX6、DAPI（下段）の免疫染色像。尚、スケールバーは1

00μmを示す。

[図10B]抗CORIN抗体でソーティングされていない細胞由来の細胞塊のマーカー発現。全細胞に対するFOXA2+/LMX1A+細胞、NURR1+細胞、およびTH+細胞の割合。

[図10C]抗CORIN抗体でソーティングされていない細胞由来の細胞塊のマーカー発現。全細胞に対するSOX1+細胞、PAX6+細胞、およびKI67+細胞の割合。

[図11]図６Ｃに示した細胞塊と同一ロットの凍結直前（28日目）の細胞塊のマーカー発現。細胞塊におけるLMX1A、FOXA2、DAPI（上段）、NURR1、FOXA2、TH、DAPI（中段）、SOX1、KI67、PAX6、DAPI（下段）の免疫染色像。スケールバーは100μmを示す。

発明を実施するための形態

[0021] 本明細書および特許請求の範囲においては、数値が「約」の用語を伴う場合、その値の±１０％の範囲を含むことを意図する。例えば、「約２０」は、「１８～２２」を含むものとする。数値の範囲は、両端点の間の全ての数値および両端点の数値を含む。範囲に関する「約」は、その範囲の両端点に適用される。従って、例えば、「約２０～３０」は、「１８～３３」を含むものとする。

[0022] 〔神経系細胞〕

本願は、立体構造を有する神経系細胞（Neural cell）を含む細胞凝集体を凍結する方法を提供する。

[0023] 神経系細胞は、神経細胞（Neuronal cell又はNeuron）、および当該神経細胞の前駆細胞、すなわち神経前駆細胞（neural progenitor cell又はneural precursor cell）等を含む。

神経系細胞は、中枢神経系の神経系細胞、又は運動神経や感覚器系の体性神経系細胞もしくは自律神経の神経系細胞の末梢神経系の神経系細胞などのあらゆる部位由来の神経系細胞であってよく、神経（ニューロン）、神経堤由来細胞、オリゴデンドロサイトもしくはアストロサイト等のグリア細胞、およびそれらの幹細胞もしくは前駆細胞等が挙げられる。神経系細胞として

、神経系細胞マーカーを発現する細胞が挙げられる。神経系細胞マーカーとしては、例えば、ＮＣＡＭ、βＩＩＩ－Ｔｕｂｕｌｉｎ（ＴＵＪ１）、チロシン水酸化酵素（ＴＨ）、セロトニン、ネスチン、ＭＡＰ２、ＭＡＰ２ＡＢ、ＮＥＵＮ、ＧＡＢＡ、グルタメート、ＣＨＡＴ、ＳＯＸ１、ＢＦ１、ＥＭＸ１、ＶＧＬＵＴ１、ＰＡＸ、ＮＫＸ、ＧＳＨ、Ｔｅｌｅｎｃｅｐｈａｌｉｎ、ＧＬＵＲ１、ＣＡＭＫＩＩ、ＣＴＩＰ２、ＴＢＲ１、Ｒｅｅｌｉｎ、ＴＢＲ１、ＢＲＮ２、ＯＴＸ２、ＬＭＸ１Ａ、ＬＭＸ１Ｂ、ＥＮ１、ＮＵＲＲ１、ＰＩＴＸ３、ＤＡＴ、ＧＩＲＫ２及びＴＨなどが挙げられるが、これらに限定されない。神経系細胞マーカーの１以上の発現により神経系細胞であることが確認できる。本明細書において、神経系細胞として、上記神経系細胞マーカーの１以上、２以上、または３以上を発現する細胞が挙げられる。

[0024] 中枢神経系の神経系細胞は、神経系細胞が存在する部位の違いにより分類できる。すなわち前脳、終脳、間脳、大脳、視床下部、中脳、後脳、中脳後脳境界領域、小脳、網膜、下垂体、又は脊髄由来の神経細胞、及びそれらの前駆細胞が挙げられる。

[0025] 前脳由来の神経細胞は、前脳組織（即ち、終脳、大脳、海馬もしくは脈絡膜、間脳、視床下部等）に存在する神経細胞である。前脳の神経細胞は、前脳神経細胞マーカーの発現により確認できる。前脳神経細胞マーカーとしては、ＯＴＸ１（前脳）、ＢＦ１（FOXG1ともいう）もしくはＳＩＸ３（終脳もしくは大脳のマーカーでもある）などが挙げられる。本明細書において、神経系細胞として、上記前脳神経細胞マーカー、終脳もしくは大脳のマーカーの１以上、２以上、または３以上を発現する細胞が挙げられる。

[0026] 大脳由来の神経細胞として、背側細胞（例えば、大脳皮質細胞、カハール・レチウス細胞、海馬神経細胞など）又は腹側細胞（例えば、大脳基底核細胞など）が挙げられる。腹側大脳神経細胞マーカーとしては、例えば、大脳基底核神経細胞マーカー（例えば、ＧＳＨ２、ＭＡＳＨ１、ＮＫＸ２．１、ＮＯＺ１）が挙げられる。背側大脳神経細胞マーカーとしては、例えば、大脳皮質神経細胞マーカー（例えば、ＰＡＸ６、ＥＭＸ１、ＴＢＲ１）が挙げ

られる。本明細書において、神経系細胞として、上記の大脳神経細胞マーカー、大脳基底核神経細胞マーカー又は大脳皮質神経細胞マーカーの１以上、２以上、または３以上を発現する細胞が挙げられる。

[0027] 中脳由来の神経系細胞としては、中脳腹側部由来神経前駆細胞、ドパミン産生神経細胞（ドパミン神経細胞又はＤｏｐａｍｉｎｅｒｇｉｃ ｎｅｕｒｏｎともいう。）又はドパミン産生神経前駆細胞（ドパミン神経前駆細胞又はＤｏｐａｍｉｎｅｒｇｉｃ ｐｒｏｇｅｎｉｔｏｒともいう。）等が挙げられる。中脳由来の神経系細胞のマーカーとしては、ＦＯＸＡ２、ＥＮ２、ＴＵＪ１等が挙げられる。ＦＯＸＡ２陽性及びＴＵＪ１陽性の神経系細胞としては、ドパミン産生神経前駆細胞及びドパミン産生神経細胞等が挙げられる。また、ドパミン産生神経細胞は、ＦＯＸＡ２陽性、ＮＵＲＲ１陽性及びＴＨ陽性であることを指標として同定することができる。

[0028] また、ドパミン産生神経前駆細胞は、ＦＯＸＡ２陽性及びＬＭＸ１Ａ陽性であることを指標として同定することができる。更に好ましくは、ＯＴＸ２、ＬＭＸ１Ａ、ＬＭＸ１Ｂ、ＣＯＲＩＮ、ＳＨＨ、ＡＡＤＣ、βＩＩＩ－Ｔｕｂｕｌｉｎ、ＥＮ１、ＮＵＲＲ１、ＰＩＴＸ３、ＤＡＴ、ＧＩＲＫ２及びＴＨのうちの１以上が陽性の細胞を含有する。本明細書において、ドパミン産生神経前駆細胞を含む細胞凝集体は、特に断りがなければ、ドパミン産生神経細胞又はドパミン作動性ニューロンなどを含んでもよい。

[0029] 本明細書において、神経系細胞として、中脳由来の神経系細胞のマーカー、ドパミン産生神経前駆細胞のマーカー又はドパミン産生神経細胞のマーカーの１以上、２以上、または３以上を発現する細胞が挙げられる。

[0030] 本明細書において、ドパミン産生神経前駆細胞として、FOXA２及び／又はＬＭＸ１Ａを発現する（ＦＯＸＡ２陽性及び／又はＬＭＸ１Ａ陽性の）細胞、好ましくは、ＦＯＸＡ２及びＬＭＸＡ１に加えて、ＯＴＸ２、ＬＭＸ１Ｂ、ＣＯＲＩＮ、ＳＨＨ、ＡＡＤＣ、及びβＩＩＩ－Ｔｕｂｕｌｉｎからなる群から選択される１以上、２以上又は３以上を発現する細胞が挙げられる。

[0031] 本明細書において、ドパミン産生神経細胞（ドパミン神経細胞）として、

ＴＨ及び／又はＮＵＲＲ１を発現する（ＴＨ陽性及び／又はＮＵＲＲ１陽性の）細胞、好ましくは、ＴＨ及びＮＵＲＲ１に加えてＦＯＸＡ２、ＡＡＤＣ、ＤＡＴ及びＧＩＲＫ２からなる群から選択される１以上、２以上又は３以上を発現する細胞が挙げられる。

[0032] 中脳後脳境界領域由来の神経細胞として、小脳、小脳板組織、脳室帯、菱脳唇等に存在する神経細胞が挙げられる。中脳後脳境界領域マーカーとしては、ＥＮ２（中脳）、ＧＢＸ２（後脳）、Ｎ－Ｃａｄｈｅｒｉｎ（中脳後脳境界領域の神経前駆細胞）が挙げられる。小脳神経前駆細胞マーカーとしては、ＧＡＢＡ作動性神経前駆細胞マーカーであるＫＩＲＲＥＬ２、ＰＴＦ１ＡもしくはＳＯＸ２や、小脳顆粒細胞前駆細胞マーカーであるＡＴＯＨ１もしくはＢＡＲＨＬ１等が挙げられる。本明細書において、神経系細胞として、上記の中脳後脳境界領域マーカー、小脳神経前駆細胞マーカー、ＧＡＢＡ作動性神経前駆細胞マーカー又は小脳顆粒細胞前駆細胞マーカーの１以上、２以上、または３以上を発現する細胞が挙げられる。

網膜由来の神経系細胞としては、視細胞、視細胞前駆細胞、網膜色素上皮細胞、角膜細胞等が挙げられる。

[0033] また、神経系細胞は、産生（分泌）する神経伝達物質の違いにより分類することもでき、例えば、ドパミン産生神経細胞、ドパミン産生神経前駆細胞、ＧＡＢＡ神経細胞、ＧＡＢＡ神経前駆細胞、コリン神経細胞、コリン神経前駆細胞、セロトニン神経細胞、セロトニン神経前駆細胞、グルタミン酸神経細胞、グルタミン酸神経前駆細胞、ノルアドレナリン神経細胞、ノルアドレナリン神経前駆細胞、アドレナリン神経細胞、アドレナリン神経前駆細胞等が挙げられる。

[0034] 運動神経や感覚器系の神経系細胞としては、コリン神経細胞又はその前駆細胞等が挙げられる。

自律神経の神経系細胞としては、コリン神経細胞、アドレナリン神経細胞、又はこれらの前駆細胞等が挙げられる。

[0035] 本明細書における神経系細胞として、好ましくは、ドパミン産生神経細胞

（ドパミン神経細胞）、ドパミン産生神経前駆細胞（ドパミン神経前駆細胞）が挙げられる。

[0036] 生体由来の神経系細胞は、ヒトなどの哺乳動物から単離した細胞であり、例えばヒト脳組織から単離した細胞としては、Nature Neuroscience,2,1137(1999)またはN. Engl. J. Med.; 344:710-9(2001)に記載されるような胎児の中脳組織に含有される細胞が例示される。

[0037] 神経系細胞はまた、胚性幹細胞（ES細胞）およびiPS細胞などの多能性幹細胞から分化誘導させて得られた細胞であってもよい。神経系細胞を多能性幹細胞から分化誘導する方法は、例えば上述の非特許文献３、４およびWO2015/034012に記載される方法（ドパミン産生神経前駆細胞）、WO2009/148170（大脳等の神経系細胞）、WO2013/065763、WO2016/013669もしくはWO2017/126551（下垂体もしくは視床下部の神経系細胞）、WO2016/039317(小脳の神経系細胞)、WO2015/076388(終脳の神経系細胞)、Numasawa-Kuroiwa, Yら、Stem Cell Reports, 2: 648-661 (2014)（神経前駆細胞）、Qiu, Lら、Stem Cells Transl Med. 6(9): 1803-1814 (2017)（ドパミン産生神経前駆細胞）が例示される。

[0038] また、神経系細胞は間葉系幹細胞（ＭＳＣ）等の複能性幹細胞から分化誘導させて得られた細胞であってもよい。神経系細胞を間葉系幹細胞から分化誘導する方法としては、J Chem Neuroanat.96:126-133(2019)に記載された方法等が例示される。

[0039] 〔多能性幹細胞〕

多能性幹細胞とは、生体に存在するほとんどすべての細胞に分化が可能である多能性を有し、かつ、増殖能を併せもつ幹細胞を意味する。多能性幹細胞は、受精卵、クローン胚、生殖幹細胞、組織内幹細胞、体細胞等から誘導することができる。多能性幹細胞には、特に限定されないが、例えば、胚性幹（ＥＳ）細胞、核移植によって得られるクローン胚由来の胚性幹（ｎｔＥＳ）細胞、精子幹細胞（ＧＳ細胞）、胚性生殖細胞（ＥＧ細胞）、人工多能性幹（ｉＰＳ）細胞、培養線維芽細胞および骨髄幹細胞由来の多能性幹細胞

（Ｍｕｓｅ細胞）などが含まれる。多能性幹細胞は、ＥＳ細胞、ｎｔＥＳ細胞、またはｉＰＳ細胞であってもよい。倫理的な点を加味すると多能性幹細胞は、ｉＰＳ細胞であってもよい。なお、胚性幹細胞は、受精１４日以内の胚から樹立されたものである。

[0040] 胚性幹細胞は、１９８１年に初めて樹立され、１９８９年以降ノックアウトマウス作製にも応用されている。１９９８年にはヒト胚性幹細胞が樹立されており、再生医学にも利用されつつある。胚性幹細胞は、内部細胞塊をフィーダー細胞上又はＬＩＦ（白血病抑制因子）を含む培地中で培養することによって製造することができる。胚性幹細胞の製造方法は、例えば、ＷＯ９６／２２３６２、ＷＯ０２／１０１０５７、ＵＳ５，８４３，７８０、ＵＳ６，２００，８０６、ＵＳ６，２８０，７１８等に記載されている。胚性幹細胞は、所定の機関から入手でき、また、市販品を購入することもできる。例えば、ヒト胚性幹細胞であるＫｈＥＳ－１、ＫｈＥＳ－２及びＫｈＥＳ－３は、京都大学再生医科学研究所から入手可能である。ヒト胚性幹細胞であるＲｘ：：ＧＦＰ株（ＫｈＥＳ－１株由来）は、国立研究開発法人理化学研究所から入手可能である。マウス胚性幹細胞であるＥＢ５細胞株及びＤ３細胞株は、それぞれ国立研究開発法人理化学研究所及びＡＴＣＣから入手可能である。

[0041] 胚性幹細胞の一つである核移植胚性幹細胞（ｎｔＥＳ細胞）は、核を取り除いた卵子に体細胞の核を移植して作ったクローン胚から樹立することができる。

[0042] ＥＧ細胞は、始原生殖細胞をｍＳＣＦ、ＬＩＦ及びｂＦＧＦを含む培地中で培養することによって製造することができる（Ｃｅｌｌ，７０：８４１－８４７，１９９２）。

[0043] 本明細書における「人工多能性幹細胞」とは、体細胞を、公知の方法等によって初期化（ｒｅｐｒｏｇｒａｍｍｉｎｇ）することで、多能性を誘導した細胞である。具体的には、線維芽細胞、又は末梢血単核球等の分化した体細胞を、ＯＣＴ３／４、ＳＯＸ２、ＫＬＦ４、ＭＹＣ（ｃ－ＭＹＣ、Ｎ－Ｍ

ＹＣ、Ｌ－ＭＹＣ）、ＧＬＩＳ１、ＮＡＮＯＧ、ＳＡＬＬ４、ＬＩＮ２８、ＥＳＲＲＢ等を含む初期化遺伝子群から選ばれる複数の遺伝子の組合せのいずれかの発現によって初期化して、多分化能を誘導した細胞が挙げられる。好ましい初期化因子の組み合わせとしては、（１）ＯＣＴ３／４、ＳＯＸ２、ＫＬＦ４、及びＭＹＣ（ｃ－ＭＹＣ又はＬ－ＭＹＣ）、（２）ＯＣＴ３／４、ＳＯＸ２、ＫＬＦ４、ＬＩＮ２８及びＬ－ＭＹＣ（Ｓｔｅｍ Ｃｅｌｌｓ，２０１３；３１：４５８－４６６）、（３）ＯＣＴ３／４、ＳＯＸ２、ＮＡＮＯＧ、ＬＩＮ２８（Ｓｃｉｅｎｃｅ ２００７； ３１８： １９１７－１９２０）等を挙げることが出来る。

[0044] 人工多能性幹細胞は、２００６年、山中らによってマウス細胞で樹立された（Ｃｅｌｌ，２００６，１２６（４），ｐｐ．６６３－６７６）。人工多能性幹細胞は、２００７年にヒト線維芽細胞でも樹立され、胚性幹細胞と同様に多能性と自己複製能を有する（Ｃｅｌｌ，２００７，１３１（５），ｐｐ．８６１－８７２；Ｓｃｉｅｎｃｅ，２００７，３１８（５８５８），ｐｐ．１９１７－１９２０；Ｎａｔ． Ｂｉｏｔｅｃｈｎｏｌ．，２００８，２６（１），ｐｐ．１０１－１０６）。

[0045] 人工多能性幹細胞は、遺伝子発現による直接初期化で製造する方法以外に、化合物の添加等によって体細胞から人工多能性幹細胞を誘導する方法によっても製造することができる（Ｓｃｉｅｎｃｅ，２０１３，３４１，ｐｐ．６５１－６５４）。

[0046] また、株化された人工多能性幹細胞を入手することも可能であり、例えば、京都大学で樹立された２０１Ｂ７細胞、２０１Ｂ７－Ｆｆ細胞、２５３Ｇ１細胞、２５３Ｇ４細胞、１２０１Ｃ１細胞、１２０５Ｄ１細胞、１２１０Ｂ２細胞、１２３１Ａ３細胞等のヒト人工多能性幹細胞細胞株が、京都大学から入手可能である。株化された人工多能性幹細胞として、例えば、京都大学で樹立されたＦｆ－ＩＰＳ１０１細胞、Ｆｆ－ＩＰＳ１０１ｓ０４細胞、ＱＨＪ－ＩＰＳ１０１及びＦｆ－ＨＤＦ１１４細胞が、京都大学から入手可能である。

[0047] 人工多能性幹細胞を製造する際に用いられる体細胞としては、特に限定は

無いが、組織由来の線維芽細胞、血球系細胞（例えば、末梢血単核球（ＰＢＭＣ）、Ｔ細胞）、肝細胞、膵臓細胞、腸上皮細胞、平滑筋細胞等が挙げられる。

[0048] 人工多能性幹細胞を製造する際に、数種類の遺伝子の発現によって初期化する場合、遺伝子を発現させるための手段は特に限定されない。上記手段としては、ウイルスベクター（例えば、レトロウイルスベクター、レンチウイルスベクター、センダイウイルスベクター、アデノウイルスベクター、又はアデノ随伴ウイルスベクター）を用いた感染法、プラスミドベクター（例えば、プラスミドベクター、又はエピソーマルベクター）を用いた遺伝子導入法（例えば、リン酸カルシウム法、リポフェクション法、レトロネクチン法、又はエレクトロポレーション法）、ＲＮＡベクターを用いた遺伝子導入法（例えば、リン酸カルシウム法、リポフェクション法、又はエレクトロポレーション法）、タンパク質の直接注入法（例えば、針を用いた方法、リポフェクション法、又はエレクトロポレーション法）等が挙げられる。

[0049] 人工多能性幹細胞は、フィーダー細胞存在下又はフィーダー細胞非存在下（フィーダーフリー）で製造できる。フィーダー細胞存在下で人工多能性幹細胞を製造する際には、公知の方法で、未分化維持因子存在下で人工多能性幹細胞を製造できる。フィーダー細胞非存在下で人工多能性幹細胞を製造する際に用いられる培地としては、特に限定は無いが、公知の胚性幹細胞及び／又は人工多能性幹細胞の維持培地、又はフィーダーフリーで人工多能性幹細胞を樹立するための培地を用いることができる。フィーダーフリーで人工多能性幹細胞を樹立するための培地としては、例えばＥｓｓｅｎｔｉａｌ８培地（Ｅ８培地）、Ｅｓｓｅｎｔｉａｌ　６培地、ＴｅＳＲ培地、ｍＴｅＳＲ培地、ｍＴｅＳＲ－Ｅ８培地、Ｓｔａｂｉｌｉｚｅｄ　Ｅｓｓｅｎｔｉａｌ　８培地、ＳｔｅｍＦｉｔ培地等のフィーダーフリー培地を挙げることができる。人工多能性幹細胞を製造する際、例えば、フィーダーフリーで体細胞に、センダイウイルスベクターを用いて、ＯＣＴ３／４、ＳＯＸ２、ＫＬＦ４、及びＭＹＣ（Ｌ－ＭＹＣ又はＣ－ＭＹＣ）の４因子を遺伝子導入す

ることで、人工多能性幹細胞を作製することができる。

[0050] 本発明に用いる多能性幹細胞は、哺乳動物の多能性幹細胞であり、好ましくはげっ歯類（例、マウス又はラット）又は霊長類（例、ヒト又はサル）の多能性幹細胞であり、より好ましくはヒト又はマウス多能性幹細胞、さらに好ましくはヒト人工多能性幹細胞（ｉＰＳ細胞）又はヒト胚性幹細胞（ＥＳ細胞）である。

[0051] 〔細胞凝集体〕

本明細書における細胞凝集体が「立体構造を有する」とは、培養細胞が、例えば浮遊培養または3次元培養などによって、細胞が相互に接着して形成される三次元の細胞集団である細胞凝集体（Cell aggregate又はsphere）を形成していることを意味する。神経系細胞の細胞凝集体を、ニューロスフェア（Neurosphere）ともいう。前記細胞凝集体の形状には特に限定はなく、球状であっても非球状であってもよい。本明細書における細胞凝集体は、好ましくは球状に近い立体的な形を有する細胞凝集体である。球状に近い立体的な形は、三次元構造を有する形であって、二次元面に投影したときに、例えば、円形又は楕円形を示す。

[0052] 立体構造を有する神経系細胞を含む細胞凝集体のサイズは特に限定されないが、通常、円相当径１５０μｍ～１０００μｍ、一態様としては例えば２００μｍ～８００μｍ、又は３００μｍ～５００μｍである。また、立体構造を有する神経系細胞を含む細胞凝集体は、通常、５００～１５００００個、一態様としては例えば１０００～１００００００個、１０００～７００００個、又は３０００～３００００個の細胞を含む。

[0053] 神経系細胞を含む細胞凝集体は、神経系細胞とともに他の細胞が含まれていても良い。神経系細胞を60％以上、70％以上、80％以上、更に好ましくは90％以上含む細胞凝集体が例示される。

[0054] 一態様として、神経系細胞を含む細胞凝集体は、ドパミン産生神経前駆細胞及び/又はドパミン産生神経細胞を６０％以上、７０％以上、又は８０％以上含んでいてもよい。すなわち、神経系細胞を含む細胞凝集体は、FOXA2、Ｌ

ＭＸ１Ａ、ＬＭＸ１Ｂ、ＮＵＲＲ１及びTHから選択される1以上のマーカーを発現する神経系細胞を、６０％以上、７０％以上、又は８０％以上含んでいてもよい。

[0055] 一態様として、神経系細胞を含む細胞凝集体は、ドパミン産生神経前駆細胞を４０％以上、６０％以上、７０％以上、８０％以上、８５％以上又は９０％以上含む。

[0056] 一態様として、神経系細胞を含む細胞凝集体は、ドパミン産生神経前駆細胞のマーカーの１以上、２以上又は３以上を発現する細胞を４０％以上、６０％以上、７０％以上、８０％以上、８５％以上又は９０％以上含む。

[0057] 一態様として、神経系細胞を含む細胞凝集体は、FOXA2陽性かつLMX1A陽性細胞を４０％以上、６０％以上、７０％以上、８０％以上、８５％以上又は９０％以上含む。一態様として、前記細胞凝集体は、更にTH陽性かつNURR１陽性の細胞を４０％以下含む。

[0058] 一態様として、神経系細胞を含む細胞凝集体は、FOXA2陽性、TH陽性、かつNURR1陽性細胞を、０％以上、１０％以上、又は２０％以上含んでいてもよい。

[0059] 一態様として、ドパミン産生神経前駆細胞を含む細胞凝集体は、NURR1陽性細胞を、６０％以下、５０％以下、４０％以下、５～５０％、５～４０％又は５～２０％含んでいてもよい。

[0060] 一態様として、ドパミン産生神経前駆細胞及び/又はドパミン産生神経細胞を含む細胞凝集体は、ＴＨ陽性細胞を、３０％以下、２０％以下、１～３０％、５～３０％、１～２０％、５～２０％、又は５～１５％含んでいてもよい。

[0061] 一態様として、ドパミン産生神経前駆細胞及び/又はドパミン産生神経細胞を含む細胞凝集体は、KI67陽性細胞を、３０％以下、１～２５％、１～２０％、又は５～２０％含んでいてもよい。

[0062] 一態様として、ドパミン産生神経前駆細胞及び/又はドパミン産生神経細胞を含む細胞凝集体は、ＳＯＸ１陽性細胞を、２０％以下、１０％以下、５％

以下、又は１％以下含んでいてもよい。

[0063] 一態様として、ドパミン産生神経前駆細胞及び/又はドパミン産生神経細胞を含む細胞凝集体は、ＰＡＸ６陽性細胞を、５％以下、２％以下、１％以下、又は０．５％以下含んでいてもよい。

[0064] 一態様として、ドパミン産生神経前駆細胞及び/又はドパミン産生神経細胞を含む細胞凝集体は、更に、TH陽性かつNURR１陽性の細胞を２０％以下、具体的には１％～２０％、より具体的には５％～１５％含む。

[0065] 一態様として、ドパミン産生神経前駆細胞及び/又はドパミン産生神経細胞を含む細胞凝集体は、FOXA2陽性かつLMX1A陽性細胞を５０％以上、好ましくは６０％以上、７０％以上、又は８０％以上含み、かつTH陽性かつNURR１陽性の細胞を２０％以下、１％～２０％、より具体的には５％～１５％含む。

[0066] 一態様として、ドパミン産生神経前駆細胞及び/又はドパミン産生神経細胞を含む細胞凝集体は、更にＳＯＸ１陽性細胞が１０％以下、好ましくは７％以下、更に好ましくは３％以下であり、ＰＡＸ６陽性細胞が５％以下、好ましくは４％以下、更に好ましくは2%以下である。

[0067] 一態様として、ドパミン産生神経前駆細胞及び/又はドパミン産生神経細胞を含む細胞凝集体は、FOXA2陽性かつLMX1A陽性細胞を６０％以上含み、かつTH陽性かつNURR１陽性の細胞を１％～２０％含み、ＳＯＸ１陽性細胞が１０％以下、好ましくは７％以下、更に好ましくは３％以下であり、ＰＡＸ６陽性細胞が５％以下、好ましくは４％以下、更に好ましくは2%以下である。

[0068] 一態様として、ドパミン産生神経前駆細胞及び/又はドパミン産生神経細胞を含む細胞凝集体は、FOXA2陽性かつLMX1A陽性の細胞を全細胞数の６０％以上含み、TH陽性かつNURR1陽性の細胞を全細胞数の２０％以下、１～２０％、又は５～１５％含んでいてもよい。

[0069] 一態様として、ドパミン産生神経前駆細胞及び/又はドパミン産生神経細胞を含む上記の細胞凝集体は、円相当径１５０μｍ～１０００μｍの細胞凝集体である。

[0070] 一態様として、ドパミン産生神経前駆細胞及び/又はドパミン産生神経細胞

を含む上記の細胞凝集体は、FOXA2陽性かつLMX1A陽性細胞を６０％以上含み、ＮＵＲＲ１陽性かつＴＨ陽性細胞を１％～２０％含み、かつ円相当径１５０μｍ～１０００μｍの細胞凝集体である。

[0071] 〔凍結方法〕

本願の方法は、（１）立体構造を有する神経系細胞を含む細胞凝集体を、凍結前に0℃以上30℃以下で保存液に接触させ、保存液に浸された細胞凝集体を調製する工程を含む。

[0072] 本願において、凍結保存液（保存液）は、凍結保護物質を含む水性液体を意味する。凍結保護物質は水分子との親和性が高く、凍結保存液中において氷晶の成長を抑制する効果の高い物質を意味し、例えば、ジメチルスルホキシド（DMSO）、エチレングリコール（EG）、プロピレングリコール（PG）、1,2-プロパンジオール（1,2-PD）、1,3-プロパンジオール（1,3-PD）、ブチレングリコール（BG）、イソプレングリコール（IPG）、ジプロピレングリコール（DPG）および／またはグリセリンなどが含まれる。本願において、凍結保護物質は好ましくはジメチルスルホキシドおよび／またはプロピレングリコールである。凍結保存液中の凍結保護物質の濃度は、凍結保護物質としてジメチルスルホキシドおよび／またはプロピレングリコールを使用する場合、通常７～１２％、好ましくは約１０％である。

[0073] 水性液体としては、例えば、生理食塩水、PBS、EBSS、HBSSなどの緩衝液やDMEM、GMEM、RPMIなどの細胞や組織などを培養する培養液、血清、血清代替物、またはこれらの混合物などを用いることができる。

[0074] また凍結保存液としては、ジメチルスルホキシド（DMSO）および／またはプロピレングリコールを実質的な成分とする市販の凍結保存液を利用することができる。凍結保存液として具体的には、STEM-CELL BANKER（SCB；ZENOAQ）、STEM-CELL BANKER DMSO free（SCB DMSO free；ZENOAQ)、Bambanker hRM（BBK；NIPPON Genetics)、CryoStor CS5（CS5；BioLife Solutions)、CryoStor CS10（CS10；BioLife Solutions)、およびSynth-a- Freeze（SaF；Thermo Fisher Scientific)などの市販の凍結保存液が挙げられる。例えば、７～１

２％、好ましくは約１０％のジメチルスルホキシドおよび／またはプロピレングリコールを含有する凍結保存液（例えば、STEM-CELL BANKER、Bambanker hRM、CryoStor CS10およびSynth-a- Freezeなど）を使用することが望ましい。

更に好ましくは、Bambanker hRMを用いることができる。

[0075] 本明細書において、細胞凝集体を凍結させる場合、凍結保存液に対する細胞数（細胞の充填密度）が８００００～５０００００００ｃｅｌｌｓ／ｍＬ、１０００００～４０００００００ｃｅｌｌｓ／ｍＬ又は２０００００～２０００００００ｃｅｌｌｓ／ｍＬ、３０００００～１０００００００ｃｅｌｌｓ／ｍＬである。

[0076] 本明細書において、細胞凝集体を凍結させる場合、細胞凝集体の円相当径は、１５０～１０００μｍ、１５０μｍ～６００μｍ、又は３００μｍ～５００μｍである。

[0077] 本明細書において、細胞凝集体及び保存液の体積は、０．２５ｍＬ～２ｍＬ、０．５ｍｌ～１．５ｍｌ、又は０．５ｍｌ～１ｍｌである。

[0078] 本明細書において、細胞凝集体及び保存液は、０．５ｍｌ～１５ｍｌ、１ｍｌ～５ｍｌ、又は１ｍｌ～２ｍｌの容器に充填されていてもよい。

[0079] 本願における凍結保存液の凝固点は特に限定されないが、通常－1℃～－10℃、好ましくは－3℃～－10℃、更に好ましくは－3℃～－6℃、更により好ましくは約－5℃である。 本明細書における凍結保存液として、実質的な成分として、７～１２％、好ましくは約１０％のジメチルスルホキシドを含み、凝固点が－１℃～－１０℃の水性液体が挙げられる。また、本明細書における凍結保存液として、実質的な成分として、７～１２％、好ましくは約１０％のジメチルスルホキシドを含み、凝固点が－３℃～－６℃の水性液体が挙げられる。

[0080] 神経系細胞を含む細胞凝集体を凍結保存液に接触させる際の温度は、通常0℃以上30℃以下、好ましくは0℃以上20℃以下、より好ましくは0℃以上10℃以下、さらにより好ましくは0℃以上4℃以下である。

[0081] また、神経系細胞を含む細胞凝集体を凍結保存液に接触させる時間は、通常５分間～２４０分間、５分間～１２０分間、好ましくは５分間～６０分間、15分間～240分間、15分間～180分間、15分間～150分間、好ましくは15分間～120分間、15分間～90分間、更に好ましくは１５分間～６０分間である。

[0082] 本願の方法はまた、（２）工程（１）で得られる保存液に浸された細胞凝集体を、少なくとも、該保存液の凝固点よりも約5℃高い温度から、凝固点よりも約5℃低い温度まで、平均２～７℃、２．５～７℃、又は3～７℃／分の温度低下速度で冷却し、凍結させる工程を含む。

[0083] 本願の方法において、保存液に浸された細胞凝集体は、少なくとも、該保存液の凝固点より約5℃高い温度から凝固点よりも約5℃低い温度に、平均２～７℃／分、２．５～７℃／分、又は平均3～7℃／分、好ましくは平均２～5.5℃／分、2.5～5.5℃／分、又は3～5.5℃／分の温度低下速度で冷却される。好ましい実施態様において、保存液に浸された細胞凝集体は、0±5℃から－30℃±5℃まで、平均２～5℃／分、２．５～５℃／分、又は3～5℃／分の温度低下速度で冷却される。

[0084] 冷却手段は上記の工程が達成される限り、特に限定されず、市販の冷凍庫を使用することができ、温度をコントロールできるプログラムフリーザー（コントロールド　レート　フリーザーとも言う）を使用してもよい。

[0085] 工程（２）において、保存液に浸された細胞凝集体は、電磁場および／または磁場に曝露されていてもよい。電磁場の周波数は、例えば約300kHz～約2MHz、好ましくは約500kHz～約1MHz、更に好ましくは約600kHz～約1MHzである。磁場の周波数は特に限定されないが、固定周波数であることが好ましい。また、例えば１０～２０００ガウス、好ましくは５０～１０００ガウス、さらに好ましくは１００～１５０ガウスの静電場下で凍結することができる。

該条件を達成する方法は特に限定されないが、例えばプロトン凍結機（株式会社菱豊フリーズシステムズ）として販売されている、電磁場および磁場を発生させる装置を備えたプログラムフリーザーを使用することができる。すなわち、磁場（Ｓｔａｔｉｃ　Ｍａｇｎｅｔｉｃ　Ｆｉｅｌｄ（ＳＭＦ）

）、電磁波（Ａｌｔｅｒｎａｔｉｎｇ　ｅｌｅｃｔｒｉｃ　ｆｉｅｌｄ（ＡＥＦ））及び超冷却気流（Ｕｌｔｒａ－ｃｏｌｄ　ａｉｒ）を併せ持つ装置を使用することができる。具体的には、３００ｋＨｚ～２ＭＨｚの電磁波を用いることで、氷形成による細胞凝集体への傷害を抑制することができる。

[0086] 本願の方法はさらに、（３）工程（２）で得られる凍結した細胞凝集体を、－50℃以下、好ましくは－80℃以下、さらに好ましくは－150℃以下に冷却する工程を含んでもよい。

[0087] 冷却手段は特に限定されないが、例えばディープフリーザー、プログラムフリーザー、プロトン凍結機、および低温の媒体（例えば、液体窒素など）に供することなどが挙げられる。

[0088] また、工程（２）または工程（３）で得られた凍結した細胞凝集体を、－80℃以下、好ましくは－150℃以下で保持し、長期保存してもよい。

[0089] 長期保存するための手段としては、例えばディープフリーザー、プログラムフリーザー、プロトン凍結機、および低温の媒体（例えば、液体窒素など）に供することなどが挙げられる。

[0090] 凍結された細胞凝集体は、適宜解凍して使用することができる。解凍方法としては、特に限定はないが、機能・活性および細胞生存率の観点から、体温程度の温度で短時間に解凍することが望ましい。具体的には３０℃～４０℃、好ましくは３５℃～３８℃、更に好ましくは、ヒトの体温付近の温度、例えば、約３７℃で解凍することが望ましい。

[0091] 本発明の方法で凍結された細胞凝集体は、解凍後に凍結保存液を培地に置換し、回復培養を行っても良いし、回復培養を行うことなく生体に移植することもできる。

[0092] すなわち、本発明の方法で凍結された細胞凝集体は、凍結を行っていない細胞凝集体と同等の性質を維持することができる。例えば、本発明の方法で凍結された細胞凝集体は、解凍後７日間回復培養を行った場合に、マーカー発現率が凍結前の細胞凝集体と同等である。例えば、ドパミン産生神経前駆細胞を含む細胞凝集体の場合、前記マーカーとしてはFOXA2、LMX1A、NURR1又

はTHを挙げることができる。ここでマーカー発現率が同等であるとは、全細胞数に対するマーカー発現細胞の割合の数値の差が、凍結前と解凍後又は解凍後7日間培養後とで、10%程度以下であることを意味する。

[0093] 本発明の方法で凍結された細胞凝集体は、回復培養を行うことなく、生体に移植可能である点で有用である。

[0094] 〔医薬組成物〕

さらに本願は、上記の方法で凍結または長期保存された細胞凝集体を有効成分として含有する医薬組成物、すなわち、移植用組成物（製剤）を提供する。

[0095] 本発明の医薬組成物（移植用組成物）は、本発明の方法で凍結された医薬組成物及び、これを解凍して得られる医薬組成物を共に含む概念である。すなわち、本発明の医薬組成物（移植用組成物）としては、神経系細胞を含む細胞凝集体及び凍結保存液を含む凍結又は非凍結の組成物、並びに、解凍後凍結保存液を投与用媒体に置換した神経系細胞を含む細胞凝集体及び投与用媒体を含む組成物が挙げられる。

[0096] 本発明の医薬組成物(移植用組成物)として、上記〔２０〕～〔２７〕に記載の移植用組成物が挙げられる。

[0097] また、一態様として、本願は、多能性幹細胞由来のドパミン産生神経前駆細胞及びドパミン産生神経細胞を６０％以上含む、円相当径１５０μｍ～１０００μｍであって、５００個～１５００００個の細胞を含む細胞凝集体、及び７％～１２％のジメチルスルホキシドおしくはプロピレングリコールを含む凝固点が－１℃～－１０℃の凍結保存液、好ましくはBambanker hRMを含み、以下の性質：

（１）解凍後に全細胞数の約６０％以上が生存している、

（２）凍結前と比較して、５０％以上の神経突起伸長活性を有する、

（３）解凍後に生存している細胞におけるFOXA2、LMX1A、NURR1およびTHの陽性率の変化が±１０％以内、好ましくは、FOXA2、LMX1A、NURR1、TH、EN1およびPITX3の陽性率の変化が±１０％以内である、

を有する凍結された移植用組成物を提供する。

[0098] 一態様として、本発明には、細胞数が８００００～５０００００００ｃｅｌｌｓ／ｍＬ、１００００００～４０００００００ｃｅｌｌｓ／ｍＬ又は２０００００～２０００００００ｃｅｌｌｓ／ｍＬ、３００００００～１０００００００ｃｅｌｌｓ／ｍＬであり、FOXA2陽性かつLMX1A陽性の細胞を全細胞数の４０％以上、好ましくは６０％以上、６０％以上、８０％以上、８５％以上又は９０％以上含み、TH陽性かつNURR1陽性の細胞を全細胞数の４０％以下、１％～２０％、又は５％～１５％含む、移植用組成物が包含される。

[0099] 一態様として、細胞凝集体の円相当径は、１５０～１０００μｍ、１５０μｍ～６００μｍ、又は３００μｍ～５００μｍである。

[0100] 一態様として、細胞凝集体及び保存液の体積は、０．２５ｍＬ～２ｍＬ、０．５ｍｌ～１．５ｍｌ、又は０．５ｍｌ～１ｍｌである。

[0101] 一態様として、細胞凝集体及び保存液は、０．５ｍｌ～１５ｍｌ、０．５ｍｌ～５ｍｌ、又は１ｍｌ～２ｍｌの容器に充填されていてもよい。

[0102] 一態様として、本発明には、解凍後に回復のための培養を行うことを要しないことを特徴とする、移植用組成物が包含される。

[0103] 一態様として、細胞凝集体を８～１９２個／ｍｌ含み、かつ、当該細胞凝集体の平均粒子径が１５０μｍ～１０００μｍであり、容器当たりの細胞数が８００００～２４０００００個である、前記〔２０〕～〔２４〕のいずれか一項に記載の移植用組成物が包含される。

[0104] 前記細胞凝集体は、神経系細胞の移植を必要とする疾患に罹患した患者のための、移植用の医薬組成物として有用であり、神経系細胞の変性、損傷もしくは機能障害を伴う疾患の治療薬等の医薬として使用することができる。すなわち、本発明の細胞凝集体、及び医薬として許容される担体を含む医薬組成物もまた、本発明の範疇である。

[0105] 神経系細胞の移植を必要とする疾患、又は神経系細胞の損傷もしくは機能障害を伴う疾患としては、例えば、脊髄損傷、運動神経疾患、多発性硬化症、筋委縮性側軸硬化症委縮性側索硬化症、ハンチントン舞踏症病、多系統萎

縮症、脊髄小脳変性症、アルツハイマー病、網膜色素変性症、加齢黄斑変性、パーキンソン症候群（パーキンソン病を含む）が挙げられる。

[0106] 本発明の一態様として、本発明のドパミン産生神経前駆細胞及び／又はドパミン産生神経細胞を含む細胞凝集体を有効成分として含有する、パーキンソン病治療用の医薬組成物が挙げられる。当該パーキンソン病治療剤に含まれるドパミン産生神経前駆細胞及び／又はドパミン産生神経細胞の細胞数は、移植片が投与後に生着できれば特に限定されないが、例えば、１回の移植あたり１．０×１０^{4}個以上含まれ得る。また、症状や体躯の大きさに合わせて適宜増減して調製されてもよい。ドパミン産生神経前駆細胞の疾患部位への移植は、例えば、Ｎａｔｕｒｅ Ｎｅｕｒｏｓｃｉｅｎｃｅ，２，１１３７（１９９９）もしくはＮ Ｅｎｇｌ Ｊ Ｍｅｄ． ；３４４：７１０－９（２００１）に記載される手法によって行うことができる。

[0107] 一態様として、本発明の医薬組成物（移植用組成物とも言う）は、ヒトに移植される神経系細胞を含む細胞凝集体及び凍結保存液を含む。本発明の医薬組成物には、凍結された固体状のものも、凍結前もしくは解凍後の液体状のものも、共に含まれる。当該医薬組成物には、凍結速度及び凍結温度に影響を与えない範囲で、細胞の生存を維持するために用いられる添加物を適宜含んでいてもよい。凍結保存液としては、上記で説明されたものが挙げられる。

[0108] 本発明の医薬組成物又は移植用組成物は、後述する通り、解凍し凍結保存液を除去し、生体に投与可能な投与用媒体に置換した後に移植に用いられる。すなわち、解凍された細胞凝集体及び投与用媒体を含む組成物もまた、本発明の医薬組成物（移植用組成物ともいう）の範疇である。

[0109] 〔移植用組成物の製造方法〕

上述の〔１〕～〔１７〕のいずれか一項に記載の凍結方法により、上述の〔２０〕～〔２７〕に記載の医薬組成物（移植用組成物）を製造することができる。すなわち本発明は、上述の医薬組成物（移植用組成物）の製造方法を包含する。

[0110] 〔治療方法〕

本発明の一態様として、本発明の細胞凝集体を、神経系細胞の移植を必要とする疾患に罹患した患者に移植する工程を含む、神経系細胞の補充を必要とする疾患の治療方法が挙げられる。

[0111] 本発明の一態様として、本発明で得られるドパミン産生神経前駆細胞および／またはドパミン産生神経細胞を含む細胞凝集体は、医薬組成物、具体的には移植用材料としてパーキンソン病患者に投与することができる。

[0112] 具体的には、本発明のドパミン産生神経前駆細胞および／またはドパミン産生神経細胞を含む細胞凝集体及び凍結保存液を含む凍結状態の医薬組成物を解凍し、適宜生理食塩水等の適切な移植用媒体に懸濁させ、患者のドパミン神経が不足している領域、例えば線条体に移植することによって行われる。例えば、該医薬組成物を解凍後に適切な担体を含む媒体で洗浄し、凍結保存液をヒトへ移植する際に細胞凝集体を懸濁するための移植用媒体に置換してもよい。解凍温度としては、特に限定はないが、上述のとおり３０℃～４０℃、好ましくは３５℃～３８℃、更に好ましくはヒトの体温付近の温度、例えば約３７℃が挙げられる。

本発明の医薬組成物（移植用組成物）に含まれる細胞凝集体は、解凍後に回復のための培養を行うことなく凍結保存液を投与用媒体に置換し、生体に移植することが可能である。

[0113] ここでドパミン産生神経前駆細胞及び／又はドパミン産生神経細胞を含む細胞凝集体に用いられる移植用媒体（投与用媒体）に用いられる担体としては、細胞の生存を維持するために用いられる物質であれば特に限定はなく当業者に周知の物質を用いることができる。具体的には、生理的な水性溶媒（生理食塩水、緩衝液、無血清培地等）を用いることができる。必要に応じて、移植医療において、移植する組織又は細胞を含む医薬組成物に、通常使用される保存剤、安定剤、還元剤、等張化剤等を配合させてもよい。

[0114] また、移植に際して、解凍した細胞凝集体を各細胞凝集体の生存能力を維持するために必要な媒体において保存してもよい。「生存能力を維持するた

めに必要な媒体」としては、培地、生理学的緩衝溶液等が挙げられるが、ドパミン産生神経前駆細胞および／またはドパミン産生神経細胞を含む細胞集団が生存する限りにおいて特に限定されず、当業者であれば適宜選択することができる。一例として、動物細胞の培養に通常用いられる培地を基礎培地として調製した培地が挙げられる。基礎培地としては、例えば、ＢＭＥ培地、ＢＧＪｂ培地、ＣＭＲＬ　１０６６培地、ＧＭＥＭ培地、Ｉｍｐｒｏｖｅｄ　ＭＥＭ　Ｚｉｎｃ　Ｏｐｔｉｏｎ培地、Ｎｅｕｒｏｂａｓａｌ培地、ＩＭＤＭ培地、Ｍｅｄｉｕｍ　１９９培地、Ｅａｇｌｅ　ＭＥＭ培地、αＭＥＭ培地、ＤＭＥＭ培地、Ｆ－１２培地、ＤＭＥＭ／Ｆ１２培地、ＩＭＤＭ／Ｆ１２培地、ハム培地、ＲＰＭＩ　１６４０培地、Ｆｉｓｃｈｅｒ’ｓ培地又はこれらの混合培地等、動物細胞の培養に用いることのできる培地を挙げることができる。

[0115] 上記細胞凝集体を移植することによって、移植されたドパミン産生神経前駆細胞および／またはドパミン産生神経細胞、及び移植後に誘導されるドパミン産生神経前駆細胞および／またはドパミン産生神経細胞は投与された患者に機能的に生着する。

[0116] ここで、本明細書における「生着」とは、移植された細胞が生体内に長期間（例：３０日以上、６０日以上、９０日以上）生存し、臓器内に接着して留まることを意味する。

[0117] 本明細書における「機能的生着」とは、移植された細胞が生着し、生体内で本来の機能を果たしている状態を意味する。

[0118] 本明細書における「機能的生着率」とは、移植した細胞のうち、機能的生着を果たした細胞の割合を意味する。移植されたドパミン産生神経前駆細胞の機能的生着率は、例えば移植片中のＴＨ陽性細胞数の計測により求めることができる。

[0119] 上記細胞凝集体を移植することによって、移植された細胞及び移植後に誘導されるドパミン産生神経前駆細胞及び／またはドパミン産生神経細胞の機能的生着率は０．１％以上、好ましくは０．２％以上、さらに好ましくは０

．４％以上、さらに好ましくは０．５％以上、さらに好ましくは０．６％以上である。

[0120] 本発明の一態様として、以下の工程を含む、ドパミン産生神経の再生を必要とする疾患の治療方法が包含される。

（１）前記〔２０〕～〔２７〕のいずれか一項に記載の移植用組成物を３０℃～４０℃、好ましくは３７℃±３℃において解凍する工程、

（２）（１）で得られた移植用組成物を、患者の線条体領域に移植する工程。

本発明の一態様として、解凍後に、培養を行うことなく、凍結保存液を投与媒体に置換し、工程（２）を行うことを特徴とする、治療方法が包含される。

[0121] 本明細書において移植の対象となる哺乳動物としては、例えば、ヒト、マウス、ラット、モルモット、ハムスター、ウサギ、ネコ、イヌ、ヒツジ、ブタ、ウシ、ウマ、ヤギ、サル等が挙げられ、好ましくはげっ歯類（例、マウス、ラット）又は霊長類（例、ヒト、サル）が、より好ましくはヒトが挙げられる。

実施例

[0122] 以下に実施例を示してさらに詳細に説明するが、本願は実施例により何ら限定されるものではない。

実施例１

実施例１の概略を、図１に示した。

[0123] ［材料と方法］

ヒトiPS細胞の維持および神経細胞への分化

Doi et al., 2014に記載のdual SMAD阻害および底板誘導プロトコルに従って、iPSC由来ドパミン産生神経前駆細胞を誘導した。すなわち、ヒトiPSC（1231A3）（京都大学）を、iMatrix511 (Nippi)でコーティングされた6ウェルプレート上においてStemFit培地(Ajinomoto)中で維持した。神経分化を開始するために、iPSCをTrypLE select (Invitrogen)と共に10分間インキュベー

トした後、単細胞に解離し、iMatrix511（Nippi)でコーティングされた6ウェルプレート上に5×10^{6}細胞／ウェルの密度で分化培地とともに播種した。分化培地は8% KSR、0.1mM MEM非必須アミノ酸（全てInvitrogen）、ピルビン酸ナトリウム(Sigma-Aldrich)および0.1mM 2-メルカプトエタノールを添加したGMEMを含む培地である。分化培地は播種翌日から12日目まで毎日交換した。播種後の細胞生存率を上げるため1日目に10μM Y-27632(Wako)を添加した。神経分化を効率的に誘導するため、LDN193189（STEMGENT)およびA83-01（Wako)を添加した。また、底板細胞を誘導するため、1～7日目に2μM パルモルファミンおよび100ng ml^{-1} FGF8（Wako)を添加し、3～12日目に3μM CHIR99021（Wako)を添加した（図１を参照）。

[0124] セルソーティングおよび培養

12日目にCORIN（発生中の脳における底板マーカー）発現細胞を単離してドパミン産生神経前駆細胞を濃縮し、ソーティング（FACS法）を行った。まず、培養細胞をPE標識抗CORIN抗体（100ng/mL；Catalent / BD）を用いて20分間染色した。死細胞およびデブリを7-AAD染色によって排除した。FACSAria IIまたはIIIセルソーターおよびFACSDivaソフトウェアプログラム(BD Biosciences)を用いて分析を行った。培養12日目にセルソーティングを行った後、ソーティングされた細胞を細胞低接着U底96ウェルプレート(Sumitomo Bakelite)上においてB27サプリメント、2μM Glutamax-I（全てInvitrogen）、10ng ml^{-1} GDNF、200mMアスコルビン酸、20ng ml^{-1} BDNF（全てWako）および400μM dbcAMP（Sigma-Aldrich)を添加したneurobasal培地を含む神経分化培地中に2-3×10^{4}細胞／ウェルの密度で再播種し、浮遊細胞塊として28日目まで培養した。また、ソーティングされていない細胞を1.5×10^{4}細胞／ウェルの密度で再播種した。培地を3日毎に半量交換し、最初の培地にのみ30μMのY-27632（Wako)を添加した。長期培養のために、浮遊細胞塊を神経分化培地中で培養した（図１を参照）。

[0125] 凍結保存法

28日目に回収した細胞塊を凍結保存し、以降の実験に用いることとした。

すなわち、表1に記載されている1mLの氷冷凍結保存液を含むクライオバイアル中に配置し、凍結するまで氷上で保持した。凍結保存のために、バイアルを冷凍コンテナ：BICELL（NIHON FREEZER）、プログラムされたフリーザー：PDF-150、250（STREX）、cryomed（ThermoFisher Scientific）またはプロトン凍結機（RYOHO FREEZER SYSTEMS）中に移行した。本実験では（図３に示される）6種類の冷却プロファイルを用いた。すなわち、BICELLをディープフリーザー（－80℃）に移行し、4時間以上保持した（図３上左）。速度が制御された凍結方法では、バイアルを0.5℃／分（図３上中央）または1℃／分（図３上右）の速度で－40℃まで凍結し、その後より速い3～5℃／分で－80℃まで冷却させた。ショッククーリング法では、－35℃まで－25℃／分の速度で凍結させ、次に－12℃まで＋10℃／分の速度で加温する工程を、凍結中の－4℃の温度において挿入した（図３下左及び中央）。プロトン凍結機で凍結したバイアルをチャンバー内で30～60分間保持した（図３下右）。プロトン凍結機は静磁場、電磁波および冷気を兼ね備えている。静磁場および電磁場は水分子の配向に影響を与え、小さな氷晶の生成を生じさせることで細胞破壊を防ぐと考えられている。しかし、これらの機構は未だ完全には理解されていない。凍結後、クライオバイアルを液体窒素タンクの気相中に保存した。凍結細胞を37℃で、約２分で解凍し、10mLのneurobasal培地を含む15mLチューブに移行した。上清を除去した後、細胞をPBSでリンスし、各アッセイまたは移植に用いた。凍結保存後の細胞数を概算するため、約50個の凝集体を解離させ、解凍の1日後および凍結前の細胞濃度を計算するために血球計算盤で計数した。

表１．本実施例で使用した市販のゼノフリー凍結保存液

[表1]

Medium	Brand name	Supplier	Mejor component
SCB	STEM-CELLBANKER	ZENOAQ	10 % DMSO
SCB DMSO free	STEM-CELLBANKER DMSO free	ZENOAQ	10 % Propylene glycol
BBK	Bambanker hRM	NIPPON Genetics	10 % DMSO
CS5	Cryostor CS5	BioLife Solutions	5 % DMSO
CS10	Cryostor CS10	BioLife Solutions	10 % DMSO
SaF	Synth-a-Freeze	Thermo Fisher Scientific	10 % DMSO

[0126] 定量RT-PCR

未分化細胞、iPSC由来ドパミン産生神経細胞（28日目、29日目、31日目,35日目）および28+0,1,3,7日目における解凍後のiPSC由来ドパミン産生神経細胞の全RNAをRNeasy Mini KitまたはRNeasy Micro Kit (Qiagen)を用いて抽出し、cDNAをSuper Script III First-Strand Synthesis System (Invitrogen)を用いて合成した。定量PCR反応をStepOneにおいてPower SYBR Green PCR Master Mix (Applied Biosystems)を用いて行った。データをデルタCt法を用いて評価し、GAPDH発現によって標準化した。使用したプライマー配列を表２に示す。

[表2]

Gene	Forward	Reverse
POU5F1	AGACCATCTGCCGCTTTGAG（配列番号１）	GCAAGGGCCGCAGCTT（配列番号２）
NANOG	GGCTCTGTTTTGCTATATCCCCTAA（配列番号３）	CATTACGATGCAGCAAATACGAGA（配列番号４）
FOXA2	TTCAGGCCCGGCTAACTCT（配列番号５）	AGTCTCGACCCCCACTTGCT（配列番号６）
LMX1A	GATCCCTTCCGACAGGGTCTC（配列番号７）	GGTTTCCCACTCTGGACTGC（配列番号８）
EN1	TGGGTGTACTGCACACGTTATTC（配列番号９）	GGAACTCCGCCTTGAGTCTCT（配列番号１０）
NURR1	CGAAACCGAAGAGCCCACAGGA（配列番号１１）	GGTCATAGCCGGGTTGGAGTCG（配列番号１２）
PITX3	GGGCCAGGAGCACAGCGACTCA（配列番号１３）	GCTGCCGCCGCTGCTTCTTTTT（配列番号１４）
TH	GCAGTTCTCGCAGGACATTG（配列番号１５）	CGGCACCATAGGCCTTCA（配列番号１６）
TPH2	TCAGCTACTTGGCAGCTCAAC（配列番号１７）	CTTGCCACTTTCGGTAGCAG（配列番号１８）
GAPDH	GGTCGGAGTCAACGGATTTG（配列番号１９）	TCAGCCTTGACGGTGCCATG（配列番号２０）

[0127] 免疫蛍光研究

インビトロ研究において、３５日目または解凍後７日目の培養細胞を4%パラホルムアルデヒドを用いて固定した。インビボ研究において、固定および凍結した脳を40μmの薄さにスライスした。薄片をフリーフローティング法を用いて免疫染色した。使用した一次抗体を表３に示す。細胞を蛍光顕微鏡(BZ-9000; Keyence)および共焦点レーザー顕微鏡(Fluoview FV1000D; Olympus)を用いて可視化した。免疫反応細胞の数を、移植片全体にわたって6片毎に定量化し、Abercrombie法を用いて補正した。

[表3]

Antibody	Species	Dilution	Supplier	Catalog#
TH	Rabbit	1:400	Millipore	AB152
NURR1	Rat	1:1000	Donated by KAN laboratory	-
FOXA2	Goat	1:500	R&D systems	AH2400
SOX1	Goat	1:100	R&D systems	AH3369
PAX6	Mouse	1:500	BD Pharmingen	561462
KI67	Rabbit	1:1000	Novocastra	NCLKi67P
TUBB3	Mouse	1:400	Covance	MMS-435P
HNA	Mouse	1:500	Millipore	MAB1281

＊TUBB３はβＩＩＩ－Ｔｕｂｕｌｉｎを意味する。

[0128] 神経突起伸長アッセイ

28日目において、神経突起伸長アッセイ用にピックアップした浮遊細胞塊をiMatrix511でコーティングされた24ウェルプレート上で5日間培養し、4%パラホルムアルデヒドで固定した。細胞塊をPE標識抗PSA-NCAM抗体(1:100; Miltenyi)で染色し、蛍光顕微鏡(BZ-9000; Keyence)を用いて可視化した。細胞体を含まないPSA-NCAM陽性神経突起の面積をPhotoshop（Adobe systems）およびWinRoof（Mitani Corporation）を用いて測定した。

[0129] 電気生理学的解析

28日目に浮遊細胞塊をパパインで解離させ、ポリ-l-オルニチン、フィブロネクチンおよびラミニン（0/F/L）でコーティングされたプレート上で49日目まで培養した。大型細胞体および神経突起様構造を有する神経を、ホールセルパッチクランプ記録のために選択した。細胞を以下の成分を含む生理食塩水溶液中に維持した：125mM NaCl、2.5mM KCl、2mM $CaCl_2$、1mM $MgCl_2$、26mM $NaHCO_3$、1.25mM NaH_2PO_4、および17mMグルコース。パッチピペットをホウケイ

酸ガラスキャピラリ(GC150TF-10; Clark)から作製した。このパッチピペットは140mM KCl、10mM HEPESおよび0.2mM EGTA（pH7.3）で構成される内液で満たした場合に、3～4MWの抵抗を有していた。電圧固定法および電流固定法での記録をパッチクランプアンプ(EPC-8; HEKA)を用いて行った。ギガシール時の抵抗は10～20GWの範囲内であった。パッチクランプアンプからの電流信号をベッセル特性を有する四極ローパスフィルター(UF-BL2; NF)に通して5kHzでフィルターにかけ、12ビットのA/Dコンバータを用いてサンプリングし、32ビットのコンピュータ(PC-9821Ra333; NEC)に保存した。全ての実験を室温で行った。

[0130] ドパミン放出アッセイ

28日目において、ドパミン放出アッセイ用にピックアップした浮遊細胞塊をO/F/Lコーティングされた12ウェルプレート上で更に28日間培養し、低濃度KCl溶液（4.7mM）で2回洗浄し、低濃度KCl溶液中で15分間インキュベートした。次に培地を高濃度KCl溶液（60mM）に15分間交換した。溶液を回収し、ドパミン濃度をLC/MS/MSで決定した。プレート上に残存していた細胞をPBS中に回収し、超音波処理した。細胞溶解物のDNA濃度をQuant-iT（商標） dsDNA Assay Kit（ThermoFisher)を用いて測定し、ドパミン濃度を補正するために使用した。

[0131] マイクロアレイ解析

cDNAマイクロアレイ解析はBio-Medical Department of Kurabo Industries Ltdにおいて行った。未分化細胞、12日目の培養細胞、iPSC由来ドパミン産生神経細胞（28日目、29日目、31日目,35日目）および28+0,1,3,7日目における解凍後のiPSC由来ドパミン産生神経細胞全RNAをGenechip（登録商標） 3'IVT pico Reagent KitおよびHuman Genome U133 Plus 2.0 array (Affymetrix)を用いて処理した。データ解析をGenechip operating software ver1.4 (Affymetrix)を用いて行った。シグナル検出および定量化をMAS5アルゴリズムを用いて行った。全プローブセットの平均シグナル強度が100と等しくなるように、グローバルノーマライゼーションを行った。$p<0.05$で、50より高いシグナル

強度を示し、サンプル間で2倍以上の変動を示すプロープセットのデータを用いて解析を実施した。

[0132] 細胞移植

実験動物を、大日本住友製薬の動物実験倫理規定、京都大学における動物実験の実施に関する規定および実験動物資源協会（ILAR; Washington, DCA）の実験動物の管理と使用に関する指針に従って治療および処理した。雄性F344/NJcl-rnu/rnu（ヌード）ラット、パーキンソン病モデルF344 NJcl -rnu/rnuラット(CLEA Japan)およびSprague Dawley（SD）ラット(SHIMIZU LABORATORY SUPPLIES)を短期間の移植研究に使用した。SDラットの右半球における内側前脳束に6-OHDAを以下の配置で注射した（A、-4.0；L、-1.3；V、-7.0）。（0.02%アスコルビン酸を含む3μLの生理食塩水中の）全19.2mgの6-OHDAをラット毎に注射した。移植の1日前から、SDラットの免疫をシクロスポリン（10mg/kg、腹腔内、LC Laboratories）を用いて毎日抑制した。細胞移植を細胞塊の定位注射を用いて行った（A、+1.0；L、-3.0；V、-5.0および-4.0；ならびにTB、0（2μl；200,000細胞／μl）。

[0133] パーキンソン病モデルヌードラットを長期間の移植研究に用いた。細胞移植を、非凍結細胞塊（A、+1.0；L、-3.0；V、-5.0および-4.0；ならびにTB、0（2μl；200,000細胞／μl）または凍結保存細胞塊（A、+1.0；L、-3.5および2.5；V、-5.5および-4.5；ならびにTB、0（4μl；200,000細胞／μl）の右脳の線条体への定位注射によって行った。実験動物を、PBSおよびその後の4%パラホルムアルデヒドを用いて経心的に麻酔および灌流した。

[0134] 行動解析

メタンフェタミン誘発回転運動評価を、ビデオでモニターされた回転用ボウルを用いて移植前、および移植後４週間毎に行った。2.5mg/kgの用量のメタンフェタミン(Dainippon Sumitomo Pharma)を腹腔内注射し、回転を90分間記録した。

[0135] 統計解析

統計解析を市販のソフトウェアパッケージ（GraphPad Prism 6; GraphPad

Software）を用いて行った。インビトロからのデータを一元配置分散分析およびテューキーの事後解析によって分析した（図２Ａ、２Ｂ、４Ａ、４Ｂ、５Aおよび図５Ｂ）。行動のデータをテューキーの多重比較検定を伴う二元配置分散分析によって分析した（図８Ａ。$p<0.05$の値の差を統計的に有意であるとみなした。行動のデータ（平均値±SEM）を除き、データを平均値±SDとして示した。

[0136] ［結果］

（１）凍結保存液の検討結果（参考例）

上述のとおりiPSC由来ドパミン産生神経前駆細胞塊に適した凍結保存条件を確立するため、上記表１に記載される臨床応用可能な凍結保存液を比較した。

[0137] まず、Doi et al, 2014に記載されるように、CORINでソーティングされていない神経前駆細胞塊を用いて凍結保存液の条件をスクリーニングした。壊死ならびにその後の遅発性アポトーシスおよび遅発性壊死の持続により、解凍後に細胞数は減少することが報告されている(Milosevic et al., 2005)。増殖能はこのプロセス中に回復し始め、細胞数は解凍の約24～48時間後に最低となることが知られている(Malpique et al., 2010; Mitchell et al., 2014; Baust et al.,2017)。また、神経毒性のある化合物または遺伝子異常に関連する疾患によって神経突起に異常が生じることも報告されている(Radio et al., 2008; Reinhardt et al., 2013; Ryan et al., 2016)。これらの現象は、神経突起の形態が神経細胞の特徴を示す良い指標となることを示唆している。そこで、細胞生存率として解凍の1日後における生存細胞の回収率を評価し、さらに神経突起伸長の面積を新たな機能評価として調べた。結果を図２Ａ、図２Ｂ及び図２Ｃに示した。

[0138] 全ての凍結保存液において、細胞生存率および神経突起伸長は凍結後に有意に減少した（図２）。10% DMSOを含む保存液は全て、STEM-CELL BANKER DMSO free (SCB DMSO free) (21±7%)およびCryoStor CS5 (CS5) (16±6%)よりも有意に高い生存率を示した（図２Ａ）。Bambanker hRM (BBK)およびSynth-

a- Freeze (SaF)は他の凍結条件よりも相対的に大きな神経突起伸長の傾向を示した（図２Ｂ及び図２Ｃ）。SCB DMSO free条件、CS5条件およびCryoStor CS10条件では、それぞれ8±17%、50±43%および13±25%の細胞塊がプレートに接着しなかった。BBKは既に日本のドラッグマスターファイルに登録されていたため、以降の実験ではBBKを用いた。

[0139] （２）中間の冷却速度および過冷却の抑制の影響

次に、BBK中のソーティングされていない細胞塊を6種類のプロトコルを用いて凍結保存した。各プロトコルの温度プロファイルを図３に示す。また、凝固点での氷核および潜熱の制御による効果を検証するため、一時的な温度降下の過程を有するショッククーリング法(Morris and Acton., 2013)についても調べた。プロトン凍結機は磁場および電磁波に加えて、特有の冷却プロファイルを有している。プロトン凍結機は過冷却を生じることなく、－4℃から－30℃まで約５℃／分で試料を冷却する。この冷却速度は、従来の緩慢法とガラス化法の中間の速度である。詳しくは、プロトン凍結機において冷却する場合、庫内の試料は、実測値として、約２～７℃／分で冷却される。

[0140] プロトン凍結機ならびにショッククーリング工程を伴わない0.5℃／分および1℃／分の条件は、他の条件よりも相対的に高い細胞生存率をもたらした（図４Ａ）。特に、プロトン凍結機および0.5℃／分での条件は非凍結細胞の場合と比較して約80%の細胞生存率を示した。また、プロトン凍結機は、ショッククーリング工程を伴う1℃／分の条件を除いて、他の凍結条件よりも著しく大きな神経突起伸長を示し、これは非凍結細胞の場合と比較して約60%の大きさであった（図４Ｂ）。これらの結果は、プロトン凍結機条件による細胞機能の維持を示している。凍結前の凍結保護物質への浸透時間を1時間に延長しても、プロトン凍結機条件では15分間での浸透時間と同様に細胞生存率および神経突起伸長の良好な結果が得られた（図５ＡおよびＢ）。また0.5℃／分の条件において細胞生存率が減少したが、その他の条件においても細胞生存率および神経突起伸長ともに有意な差は観察されなかった（図５ＡおよびＢ）。以上より、中間の冷却速度および過冷却の抑制がiPSC由来ドパミン

産生神経前駆細胞塊の凍結保存に有効であることがわかった。

上記で用いた細胞凝集塊は、抗CORIN抗体によるソーティングを行わないこと以外は、図１と同様の方法で分化誘導を行った。凍結前の当該細胞凝集塊のマーカー発現を測定した結果を図１０及び表４に示した。

[表4]

	DAPI陽性細胞に対する割合
FOXA2/LMX1A	８４．９±５．５％
NURR1	２１．２±１１．３％
TH	７．４±２．７％
SOX1	４．８±２．０％
PAX6	２．６±１．０％
KI67	８．５±１．８％

[0141] （３）凍結保存したiPSC由来ドパミン産生神経前駆細胞塊の細胞数および機能

上記のスクリーニング結果に基づき、BBKおよびプロトン凍結機を用いて凍結保存したiPSC由来ドパミン産生神経前駆細胞塊の解凍後の性質を調べた。iPSC由来ドパミン産生神経前駆細胞を80％以上に濃縮するためにCORIN抗体を用いてソーティングし、上述した条件で浮遊培養を行い、細胞凝集体を形成させた後、BBKおよびプロトン凍結機を用いて凍結保存した（図１を参照）。BBKの浸透時間は1時間であった。

[0142] その結果、凍結保存した細胞塊の大きさは２００μｍ～５００μｍであり、細胞数５５００個～１２０００個であった（細胞数については、全細胞塊で計測していないため、細胞塊の大きさから２０００個～１５０００個と推定される）。得られた細胞塊は52±8%の細胞生存率および51±19%の神経突起伸長を示した（図６Ａ、Ｂ）。ドパミン産生神経前駆細胞であることを確認するため、タンパク質マーカーおよび遺伝子発現を解凍の7日後に調べた。免疫細胞化学の結果、凍結保存細胞塊は非凍結細胞塊と比較してほとんど同じレベルのドパミン産生神経前駆細胞マーカー（FOXA2）、ドパミン産生神経マーカー（NURR1およびTH）、および増殖性細胞マーカー（KI67）を発現していたことが分かった（図６Ｃ～Ｅ）。

細胞ソーティングのプロセスによってドパミン産生神経前駆細胞が高度に濃縮されていたため、神経幹細胞マーカー（SOX1およびPAX6）を発現する細胞は非凍結細胞塊および凍結細胞塊の両方において極めて少なかった。これは凍結保存プロセスによって異常増殖が生じなかったことを示している。さらにqPCR分析により、ドパミン産生神経前駆細胞マーカー（FOXA2、LMX1AおよびEN1）およびドパミン産生神経マーカー（NURR1、PITX3およびTH）の発現レベルが凍結保存の有無によって変化しなかったことが分かった（図６Ｇ）。多能性マーカー（POU5F1およびNANOG）の発現レベルは、両方の細胞塊において0日目の1%未満に維持された（図６Ｆ）。

[0143] また、図６Cに示した細胞塊と同一ロットの凍結直前の細胞塊のマーカー発現を図１１に、複数ロットにおいてマーカー発現量を測定した結果を表５にそれぞれ示した。

[表5]

Lot	1	2	3	4	平均	SD
SOX1	0.0	0.1	1.1	0.0	0.3	0.5
PAX6	0.0	0.0	0.0	0.5	0.1	0.3
KI67	20.0	20.1	7.7	16.9	16.2	5.8
NURR1	27.8	38.9	8.5	40.8	29.0	14.8
TH	13.3	12.4	9.6	12.0	11.8	1.6

また、FOXA2及びLMX1Aの発現率を比較した（表６）。

[表6]

LOT	1	2	3	4	平均	SD
LMX1A	86.5	87.8	64.8	92.6	82.9	12.4
FOXA2	80.9	88.1	70.3	90.8	82.5	9.1
LMX1A/FOXA2	78.5	82.6	57.7	85.5	76.1	12.6

[0144] また、凍結保存／解凍プロセス後の成熟化の時間変化を概観するため、マイクロアレイデータを用いたグローバルPCA分析を行った。有意な信号を含む全21,882種のプローブセットを分析に用いた。PC1およびPC2の寄与はそれぞれ48.0%および15.7%であった。PCスコアプロットは3つの主要な群（0日目（i

PSC）、12日目、および28日目以降）にクラスター化された（図６Ｈ）。非凍結試料および凍結保存試料のPCAプロットは共に、28日目の凍結保存および28日目以降に関わらず、PC1軸およびPC2軸の正の方向に移動していた。同一の分化バッチの非凍結試料と凍結保存試料との間において、23349種のプローブセットのうち206種（0.88%）のみが2倍を超える増加または減少を示した。一方、異なるバッチの非凍結試料間において、23275種のプローブセットのうち222種（0.95%）が2倍を超える増加または減少を示した（図６Ｉ、Ｊ）。この結果は、凍結保存プロセスがドパミン産生神経前駆細胞の細胞特性に影響を与えていないことを強く示している。

[0145] iPSC由来ドパミン産生神経前駆細胞塊の機能的成熟を確認するため、電気生理学的解析およびドパミン放出測定を行った。凍結保存細胞塊を解離させ、さらなる成熟化のためにプレート上で培養すると、ほとんどの細胞は28＋21日目に$TH^+/TUBB3^+$ ドパミン産生神経を生じた（図６Ｋ）。この時点において、成熟ドパミン産生神経由来の連続した活動電位を電流固定化のホールセルパッチクランプ法で検出した（図６Ｌ）。また、56日目の非凍結細胞塊および28＋28日目の凍結保存細胞塊のドパミン分泌をLC/MS/MSで検出した。放出されたドパミンの量は非凍結細胞塊と同程度であった（図６Ｍ）。以上より、凍結保存したiPSC由来ドパミン産生神経前駆細胞塊の細胞数および機能は維持されていることがわかった。

[0146] （４）凍結保存したiPSC由来ドパミン産生神経前駆細胞塊の生着、及び6-OHDA病変ラットの行動

凍結保存したiPSC由来ドパミン産生神経前駆細胞が移植後に生存可能であることを確認するため、非凍結細胞塊および凍結保存細胞塊を数種類のラット線条体に移植し、短期間観察した後、移植片生着率および生存細胞数を計数した。結果を表７に示す。両群の間で移植片が生着したラットの数に差はなかった。プロトン凍結機およびBBKを用いて凍結保存した細胞塊を移植した場合、全生存細胞（HNA^+）の割合は非凍結細胞の場合の42±20%であった。

表７．凍結保存細胞塊の移植片生着率を評価した短期間の検証実験の結果。

[表7]

	Animal		Duration	Number of animals with cells		Average number of survived cells	
	strain	Model		Unfrozen	Frozen	Unfrozen	Frozen
1383D6	Nude	Nomal	1 month	3/3	3/3	25555	4554
1231A3	Nude	Nomal	1 month	2/3	2/3	13589	8330
FF-I01	SD	6-OHDA lesioned	3 months	2/2	1/1	31781	18326
FF-I01	Nude	6-OHDA lesioned	2 weeks	3/3	3/3	31685	11520

[0147] 移植を用いた短期間での検証実験の結果として、インビボにおいて非凍結細胞に類似する細胞生存数を達成するためには、非凍結細胞の2倍を超える凍結保存細胞を移植すべきであることが分かった。そこで凍結保存細胞の生存数および薬理効果を評価するため、非凍結細胞塊（4×10^5細胞）および凍結保存細胞塊（8×10^5細胞）を6-OHDA病変PD疾患モデルラットに移植し、メタンフェタミン誘発回転運動を測定した。移植の24週間後、両群において異常回転が減少した（図８Ａ）。24週間目における免疫蛍光染色により、行動回復を示した全てのラットで細胞の生着が確認された。非凍結細胞塊由来の移植片において55996±3603個のHNA^+細胞が生存し、凍結保存細胞塊由来の移植片において36486±3578個のHNA^+細胞が生存していた（図８Ｂ、Ｃ）。HNA^+細胞生存数について、両群間に有意な差は見出されなかった。両群において、TH^+細胞は生存し、正常な神経突起伸長を示した（図８Ｄ～Ｇ）。非凍結細胞塊由来の移植片と凍結保存細胞塊由来の移植片との間でTH^+細胞の数に差はなかった（3603±1576細胞／移植片と3578±1490細胞／移植片）（図８Ｈ）。非凍結細胞塊および凍結保存細胞塊に由来する移植片における生存TH^+細胞は、それぞれ注入した細胞の1.1±0.4%および0.5±0.2%であった。この結果は、非凍結細胞塊の場合と比較して約50%のTH^+神経が凍結保存細胞塊由来の移植片において生存していたことを意味する。さらに、両群の生存細胞の大部分は$FOXA2^+$ ドパミン産生神経前駆細胞であった：非凍結細胞塊の71±6%、および凍結保存細胞塊の76±4%（図８Ｉ、Ｊ）。一方、異常増殖のリスクを生じ得る$KI67^+$増殖性細胞は両群においてほとんど認められなかった（図８Ｉ、Ｋ）。以上のことから、凍結保存したiPSC由来ドパミン産生神経前駆細胞塊は生

着し、6-OHDA病変ラットの行動を改善することがわかった。

[0148] （５）ドパミン産生神経前駆細胞マーカーの発現

解凍後の細胞が非凍結細胞と同様にドパミン産生神経細胞への成熟能を有することを確認するため、タンパク質マーカーおよび遺伝子発現を解凍の7日後まで経時的に調べた。免疫細胞化学の結果、凍結保存細胞塊は非凍結細胞塊と比較してほとんど同じレベルのFOXA2を発現し続けることがわかった（図７Ａ）。非凍結細胞においてNURR1+細胞は28日目から35日目にかけて増加する。解凍後の細胞は非凍結細胞にやや遅れるものの解凍後7日目ではNURR1+細胞の増加が認められた（図７Ｂ）。さらにqPCR分析により、非凍結細胞ではTHの発現レベルが35日目まで培養期間に伴い上昇することがわかった。解凍後0日目から3日目の細胞はTHの発現が一定のままであるが、7日目にはTHの発現が増加することが確認された（図７Ｃ）。以上のことから、凍結保存したiPSC由来ドパミン産生神経前駆細胞塊はドパミン産生神経細胞への成熟能を保持していることがわかった。

また、複数回のロットで、非凍結細胞を35日目まで培養した場合と、28日目に凍結し、解凍後７日間培養した場合を比較した結果を以下の表８に示した。

[表8]

LOT		1	2	3	4	平均	SD
D35	SOX1	0.5	0.0	0.0	0.3	0.2	0.2
	PAX6	0.0	0.0	0.4	0.2	0.1	0.2
	KI67	10.4	6.1	4.3	8.4	7.3	2.7
	FOXA2	82.9	90.4	83.0	88.8	86.3	3.9
	NURR1	36.8	51.3	9.5	28.5	31.5	17.5
	TH	27.0	18.4	18.8	15.8	20.0	4.9
D28+7	SOX1	0.0	0.4	0.0	0.0	0.1	0.2
	PAX6	0.0	0.0	0.5	0.0	0.1	0.3
	KI67	13.3	7.5	16.9	4.6	10.6	5.5
	FOXA2	83.2	88.8	81.1	82.0	83.8	3.5
	NURR1	44.7	41.0	23.9	46.1	38.9	10.2
	TH	15.9	9.0	23.3	20.2	17.1	6.2

[0149] （６）解凍条件の比較（参考）

BBKを凍結保存液に用い、プロトンフリーザーで凍結させた凍結保存細胞塊の解凍条件について、検討した。液体窒素気層で保管されていたチューブを取り出し、条件(i)室温、(ii)37℃湯浴、(iii)プログラムフリーザーを使用して約3℃/min(実測値)で融解、の３条件で解凍した。解凍に要した時間はそれぞれ(i)１７分間、(ii)２分間、(iii)約２５分間であった。解凍後の細胞塊の生存率（生細胞回収率）及び神経突起伸長活性を上述の方法で測定した。図９に示すとおり、３７℃湯浴での解凍が、最も良好な結果を与えた。

請求の範囲

[請求項1] 以下の工程（１）及び工程（２）を含む、立体構造を有する神経系細胞を含む細胞凝集体を凍結する方法：

（１）立体構造を有する神経系細胞を含む細胞凝集体を、凍結前に０℃以上３０℃以下で、保存液に接触させ、保存液に浸された細胞凝集体を調製する工程、及び；

（２）工程（１）で得られる保存液に浸された細胞凝集体を、少なくとも、該保存液の凝固点よりも約５℃高い温度から、凝固点よりも約５℃低い温度まで、平均２～７℃／分の温度低下速度で冷却し、凍結させる工程。

[請求項2] 工程（２）における温度低下速度が、平均３～７℃／分である、請求項１に記載の方法。

[請求項3] 工程（１）において、細胞凝集体を１５分間～９０分間、好ましくは１５分間～６０分間保存液に接触させる、請求項１又は２に記載の方法。

[請求項4] 保存液の凝固点が、－１℃～－１０℃である、請求項１～３のいずれか一項に記載の方法。

[請求項5] 保存液が、７%～１２%のジメチルスルホキシド及び/又はプロピレングリコールを含む水性液体であり、工程（２）が、０±５℃から－３０±５℃まで、平均２～５℃／分の温度低下速度で冷却する工程である、請求項１～４のいずれか一項に記載の方法。

[請求項6] 工程（２）における温度低下速度が、平均３～５℃／分である、請求項１～５のいずれか一項に記載の方法。

[請求項7] 更に、以下の工程（３）を含む、請求項１～６のいずれか一項に記載の方法：

（３）工程（２）で得られる凍結した細胞凝集体を、－５０℃以下に冷却する工程。

[請求項8] 神経系細胞を含む細胞凝集体が、多能性幹細胞由来の神経系細胞を

含む細胞凝集体である、請求項１～７のいずれか一項に記載の方法。

[請求項9] 神経系細胞を含む細胞凝集体が、FOXA2、TH及びNURR1の少なくとも１つが陽性の細胞を含む、請求項１～８のいずれか一項に記載の方法。

[請求項10] 神経系細胞を含む細胞凝集体が、FOXA2陽性かつLMX1A陽性の細胞を含む、請求項９に記載の方法。

[請求項11] 神経系細胞を含む細胞凝集体が、FOXA2陽性、TH陽性、かつNURR1陽性細胞を含む、請求項９に記載の方法。

[請求項12] 神経系細胞を含む細胞凝集体が、FOXA2陽性かつLMX1A陽性の細胞を全細胞数の４０％以上含み、TH陽性かつNURR1陽性の細胞を全細胞数の４０％以下含む、請求項１～８のいずれか一項に記載の方法。

[請求項13] 神経系細胞を含む細胞凝集体が、ドパミン産生神経前駆細胞及び／又はドパミン産生神経細胞を含む、請求項１～１２のいずれか一項に記載の方法。

[請求項14] 細胞凝集体が、５００個～１５００００個の細胞を含む、請求項１～１３のいずれか一項に記載の方法。

[請求項15] 保存液中に含まれる細胞数が８００００～５０００００００ｃｅｌｌｓ／ｍＬであり、細胞凝集体の円相当径が１５０～１０００μｍである、請求項１～１４のいずれか一項に記載の方法。

[請求項16] 細胞凝集体及び保存液の体積が０．２５ｍＬ～２ｍＬである、請求項１～１５のいずれか一項に記載の方法。

[請求項17] 細胞凝集体及び保存液が、０．５ｍｌ～１５ｍｌの容器に充填されている、請求項１～１６のいずれか一項に記載の方法。

[請求項18] 請求項１～１７のいずれかに記載の方法で得られる凍結した細胞凝集体を、－８０℃以下で保持することを含む、立体構造を有する神経系細胞を含む細胞凝集体の長期保存方法。

[請求項19] 解凍後に回復のための培養を行うことを要しない、凍結された細胞凝集体を得ることを特徴とする、請求項１～１８のいずれかに記載の

方法。

[請求項20] 請求項１～１９のいずれか一項に記載の方法で凍結または長期保存された細胞凝集体を有効成分として含有する移植用組成物。

[請求項21] 多能性幹細胞由来のドパミン産生神経前駆細胞及びドパミン産生神経細胞を６０％以上含む、円相当径１５０μｍ～１０００μｍであって、５００個～１５００００個の細胞を含む細胞凝集体、及び７％～１２％のジメチルスルホキシドもしくはプロピレングリコールを含む凝固点が－１℃～－１０℃の凍結保存液を含み、以下の性質：

（１）解凍後に全細胞数の約６０％以上が生存している、

（２）凍結前と比較して、５０％以上の神経突起伸長活性を有する、

（３）解凍後に生存している細胞におけるFOXA2、LMX1A、NURR1およびTHの陽性率の変化が±１０％以内である、を示す、凍結された移植用組成物。

[請求項22] 細胞数が８００００～５０００００００ｃｅｌｌｓ／ｍＬであり、FOXA2陽性かつLMX1A陽性の細胞を全細胞数の４０％以上含み、TH陽性かつNURR1陽性の細胞を全細胞数の４０％以下含む、請求項２０又は２１に記載の移植用組成物。

[請求項23] 細胞凝集体の円相当径が１５０～１０００μｍである、請求項２０～２２のいずれか一項に記載の移植用組成物。

[請求項24] 解凍後に回復のための培養を行うことを要しないことを特徴とする、請求項２０～２３のいずれか一項に記載の移植用組成物。

[請求項25] 細胞凝集体を８～１９２個／ｍｌ含み、かつ、当該細胞凝集体の円相当径が１５０μｍ～１０００μｍであり、容器当たりの細胞数が８００００～２４０００００個である、請求項２０～２４のいずれか一項に記載の移植用組成物。

[請求項26] 細胞凝集体及び保存液の体積が０．２５ｍＬ～２ｍＬである、請求項２０～２５のいずれか一項に記載の移植用組成物。

[請求項27] ０．５ｍｌ～１５ｍｌの容器に充填されている、請求項２０～２６

のいずれか一項に記載の移植用組成物。

[請求項28] 請求項１～１７のいずれか一項に記載の方法で、細胞数が８０００００～５０００００００ｃｅｌｌｓ／ｍＬであり、FOXA2陽性かつLMX1A陽性の細胞を全細胞数の４０％以上含み、TH陽性かつNURR1陽性の細胞を全細胞数の４０％以下含む、円相当径１５０μｍ～１０００μｍの細胞凝集体を凍結することを含む、ドパミン産生神経前駆細胞を有効成分として含有する移植用組成物の製造方法。

[請求項29] 当該細胞凝集体の円相当径が１５０～１０００μｍである細胞凝集体の集団を含み、容器当たりの細胞数が８００００～２４０００００個であり、細胞凝集体及び保存液の体積が０．２５ｍＬ～２ｍＬである、０．５ｍｌ～１５ｍｌの容器に充填されている、神経系細胞の凝集体を有効成分として含有する請求項２８に記載の移植用組成物の製造方法。

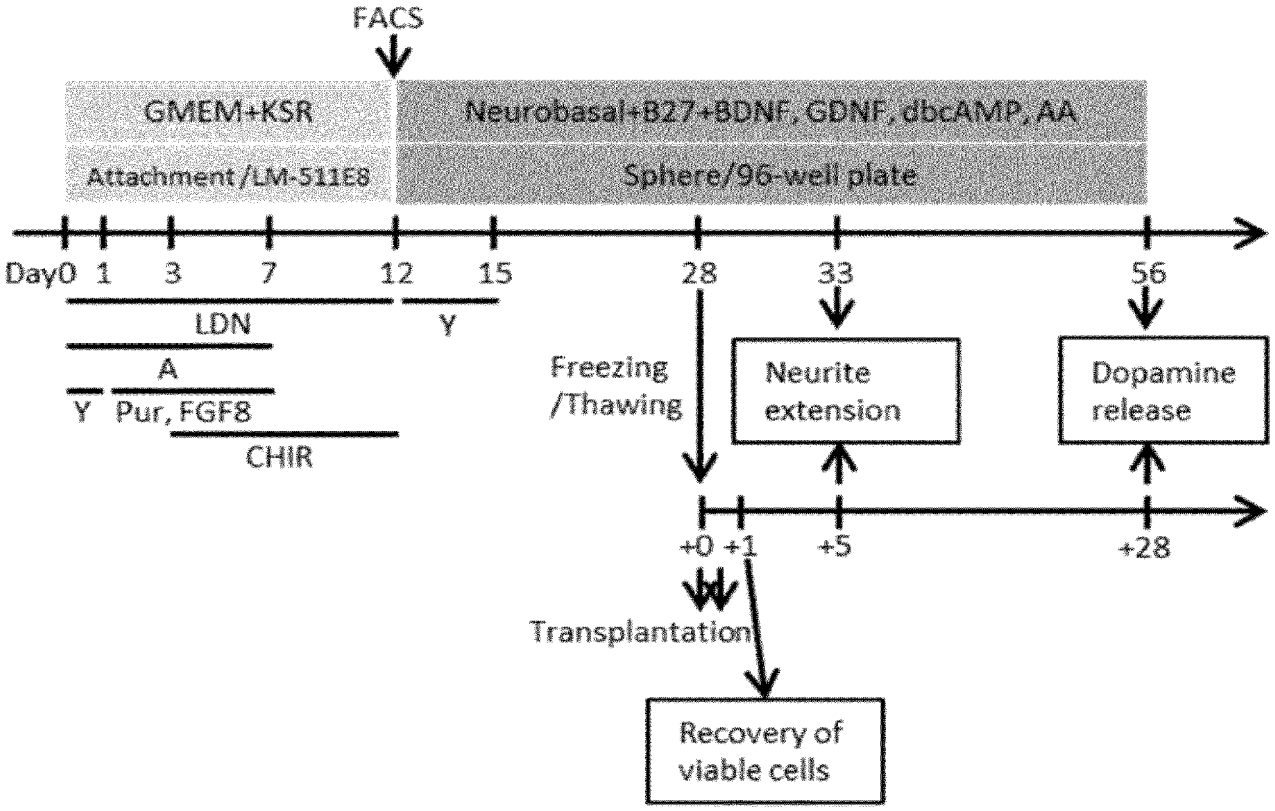
[請求項30] 以下の工程を含む、ドパミン産生神経の再生を必要とする疾患の治療方法：

（１）請求項２０～２７のいずれか一項に記載の移植用組成物を３０℃～４０℃、好ましくは３７℃±３℃において解凍する工程、

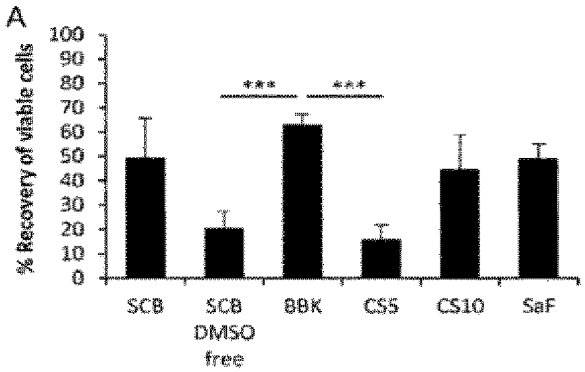
（２）（１）で得られた移植用組成物を、患者の線条体領域に移植する工程。

[請求項31] 解凍後に、培養を行うことなく、凍結保存液を投与媒体に置換し、工程（２）を行うことを特徴とする、請求項３０に記載の方法。

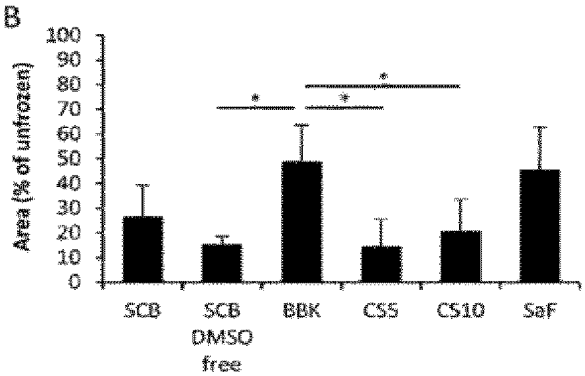
[図1]


FACS
GMEM+KSR
Neurobasal+B27+BDNF, GDNF, dbcAMP, AA
Attachment/LM-511E8
Sphere/96-well plate
Day0 1 3 7 12 15 28 33 56
LDN
Y
A
Y Pur, FGF8
CHIR
Freezing /Thawing
Neurite extension
Dopamine release
+0 +1 +5 +28
Transplantation
Recovery of viable cells

[図2A]

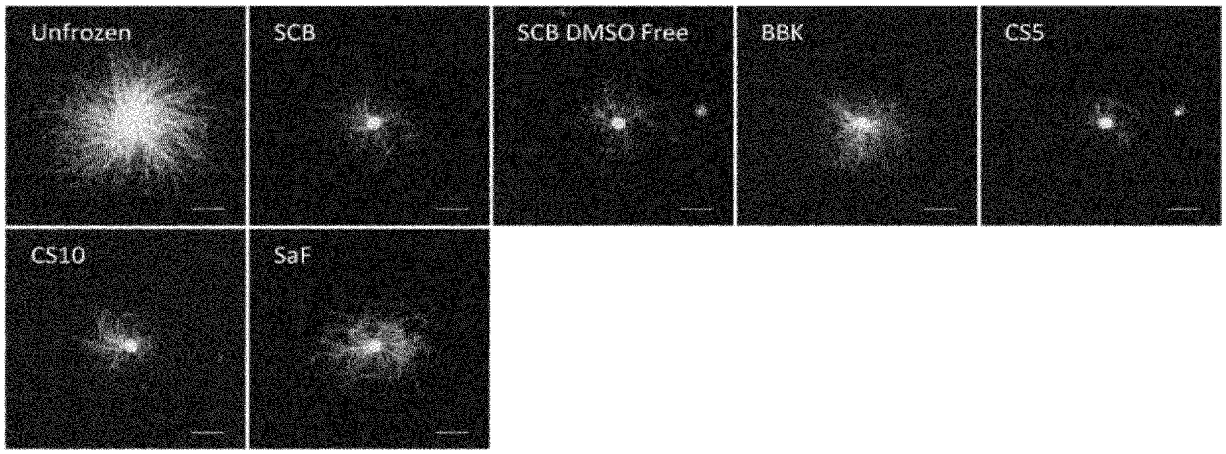

A
% Recovery of viable cells
100
90
80
70
60
50
40
30
20
10
0
***
***
SCB
SCB DMSO free
BBK
CS5
CS10
SaF

[図2B]

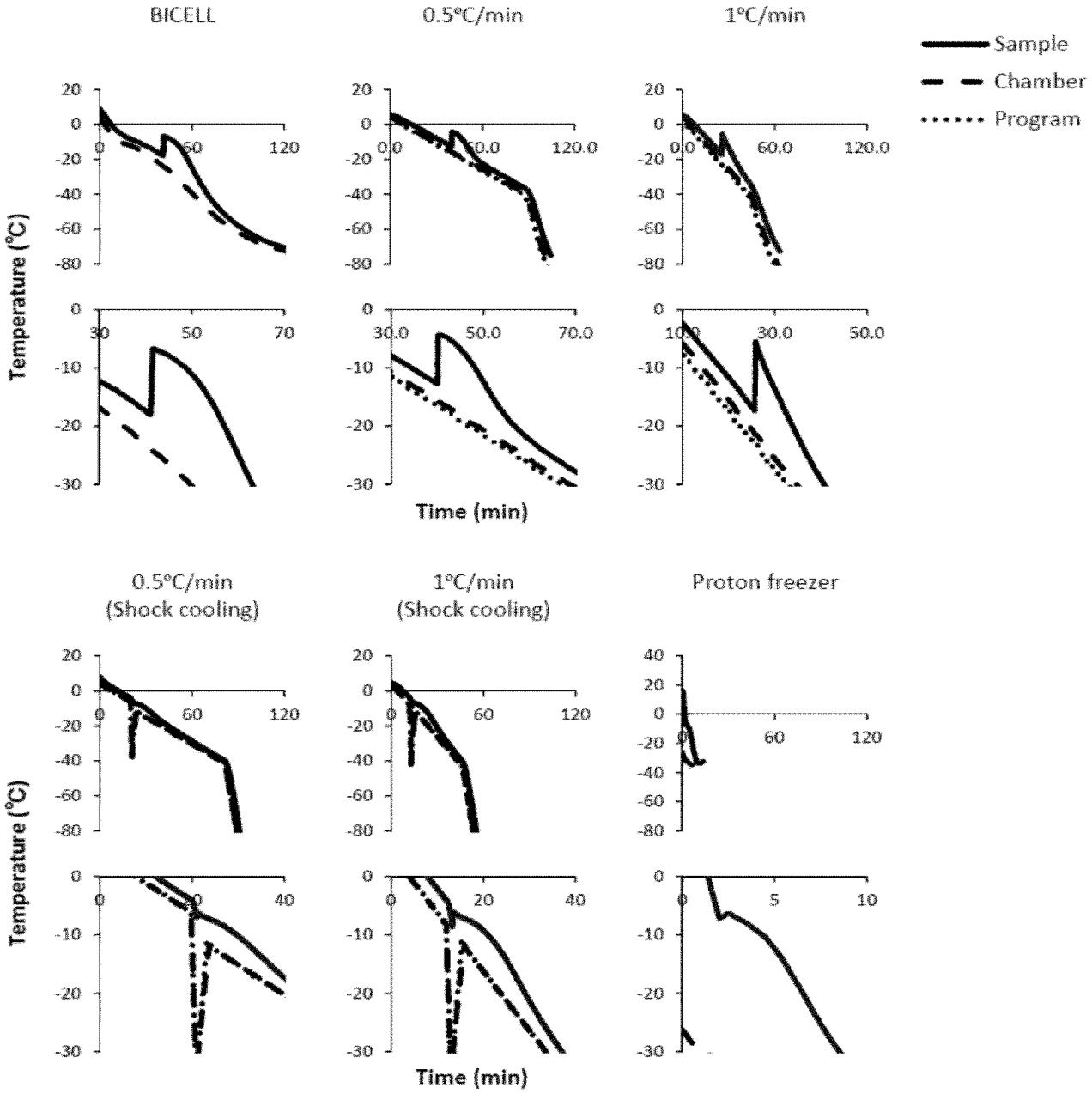

B
Area (% of unfrozen)
100
90
80
70
60
50
40
30
20
10
0
*
*
*
SCB
SCB DMSO free
BBK
CS5
CS10
SaF

[図2C]

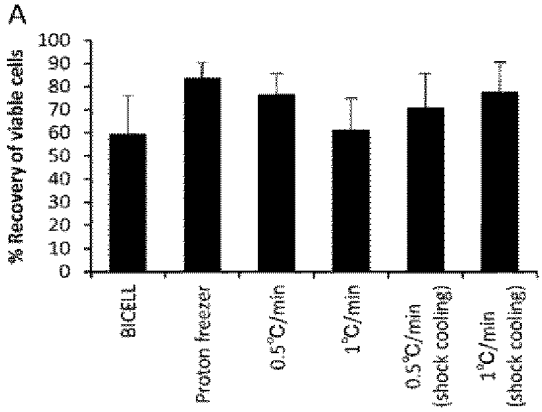
C


Unfrozen
SCB
SCB DMSO Free
BBK
CS5
CS10
SaF

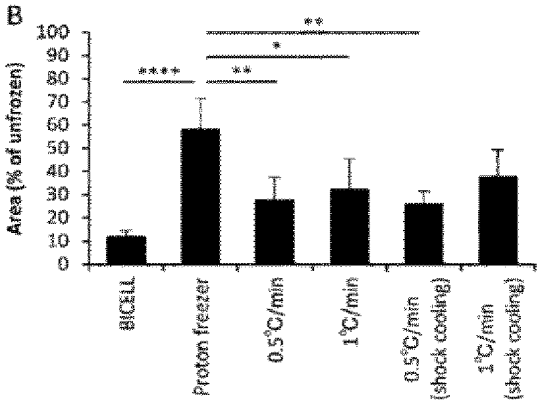
[図3]


BICELL
0.5℃/min
1℃/min
Sample
Chamber
Program
Temperature (℃)
Time (min)
0.5℃/min (Shock cooling)
1℃/min (Shock cooling)
Proton freezer

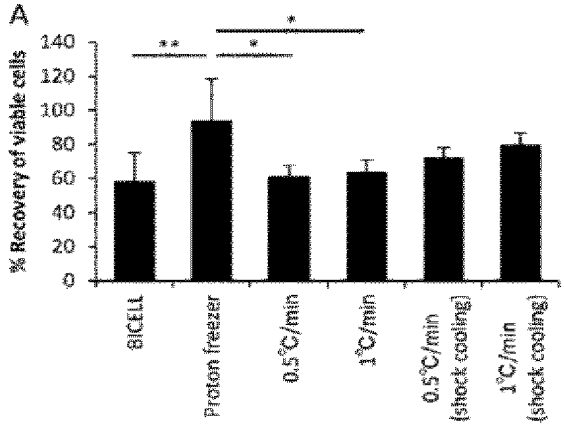
[図4A]



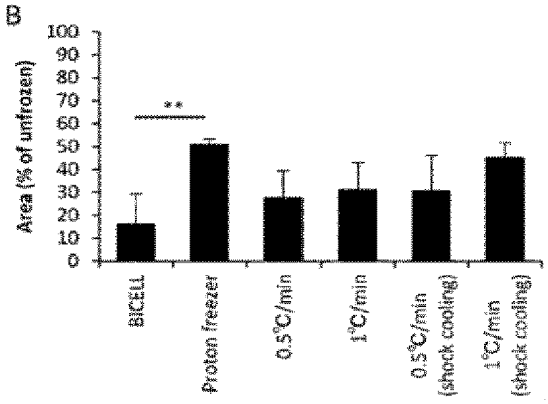
[図4B]



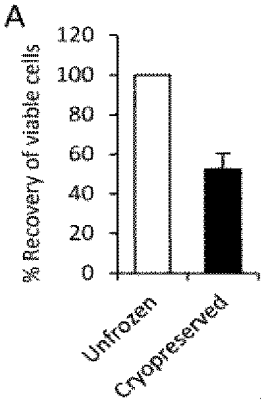
[図5A]



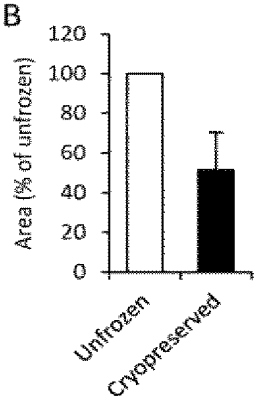
[図5B]



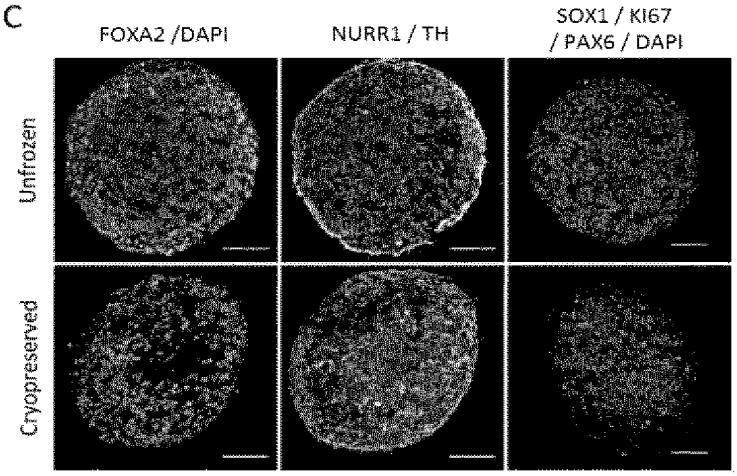
[図6A]



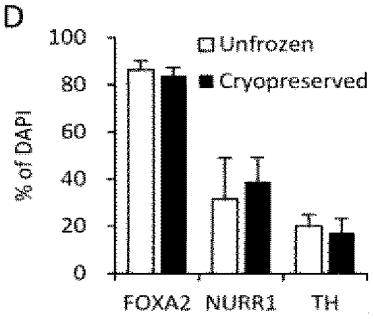
[図6B]



[図6C]

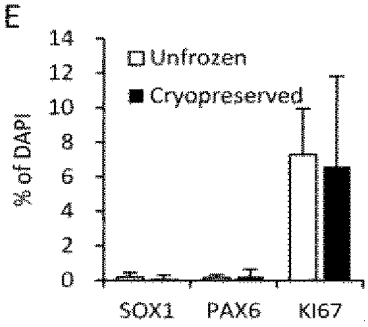


[図6D]



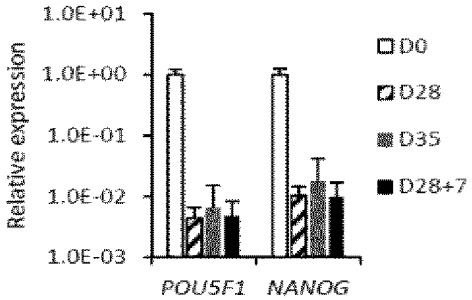
[図6E]

E



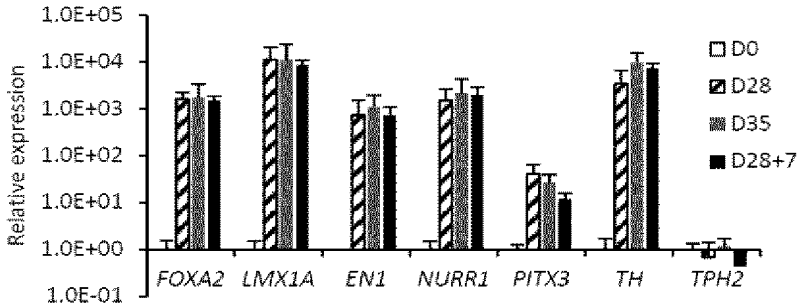
[図6F]

F



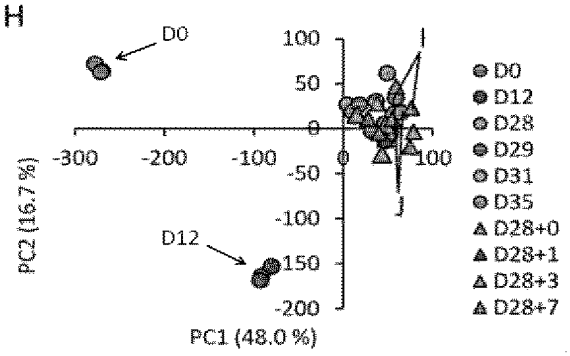
[図6G]

G



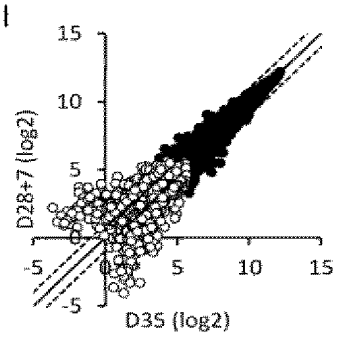
[図6H]

H



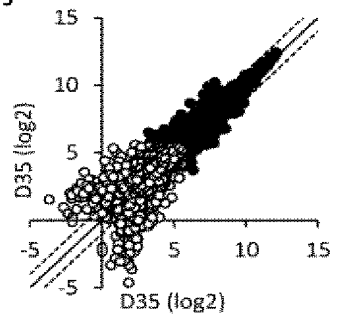
[図6I]

I



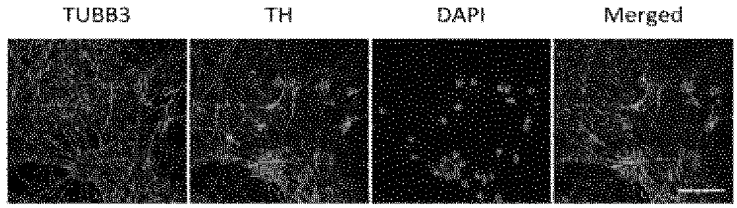
[図6J]

J



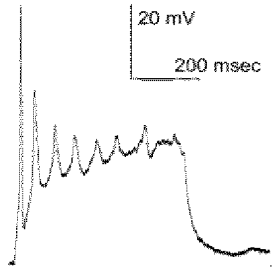
[図6K]

K



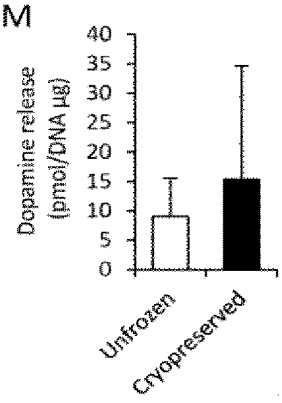
[図6L]

L

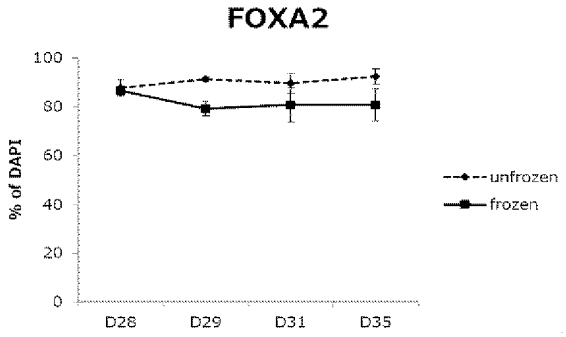


[図6M]

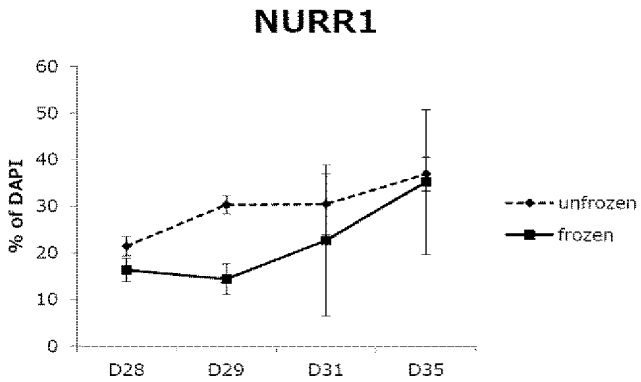
M



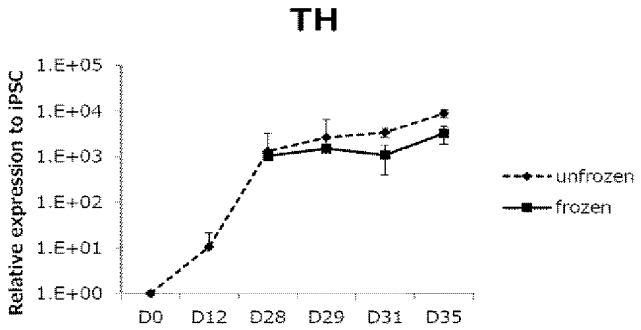
[図7A]



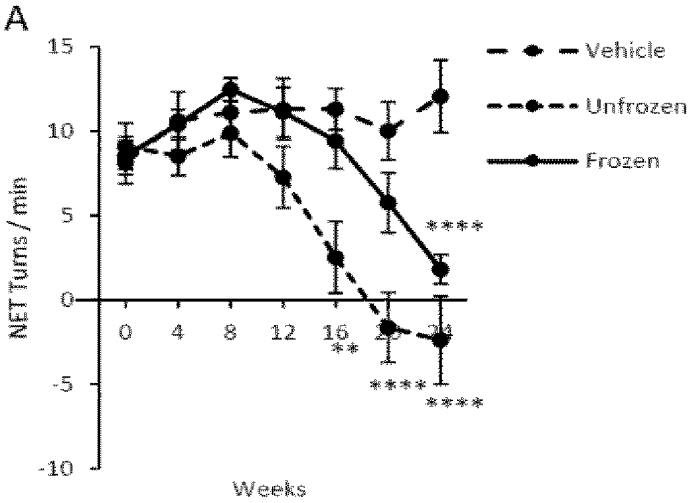
[図7B]



[図7C]

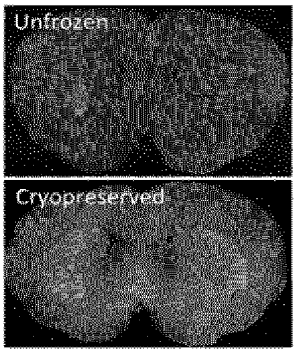


[図8A]



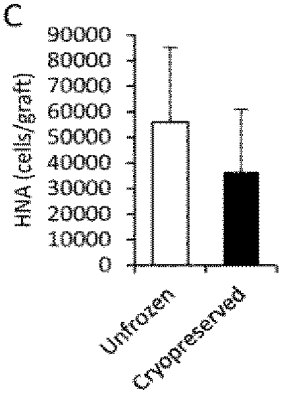
[図8B]

B



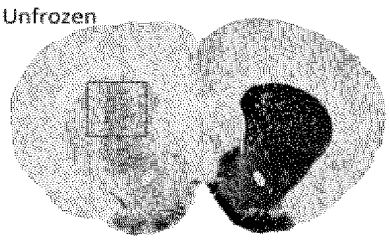
[図8C]

C



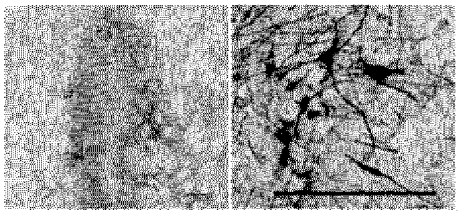
[図8D]

D



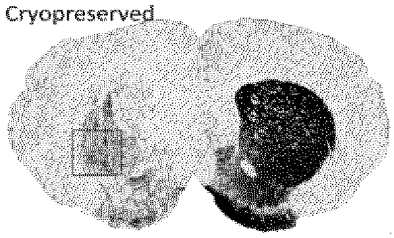
[図8E]

E



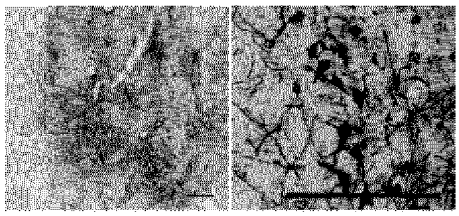
[図8F]

F



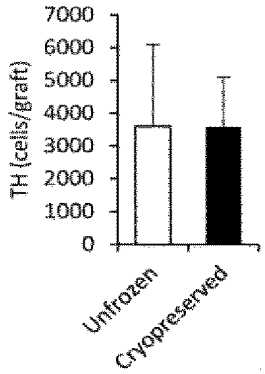
[図8G]

G



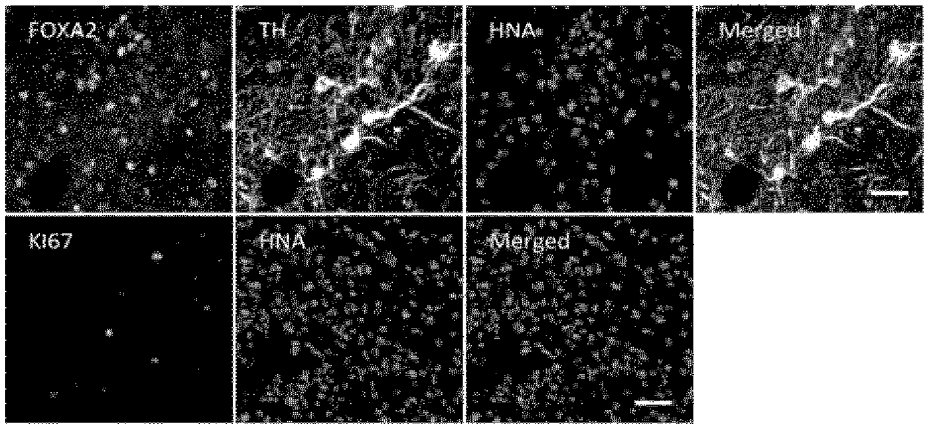
[図8H]

H



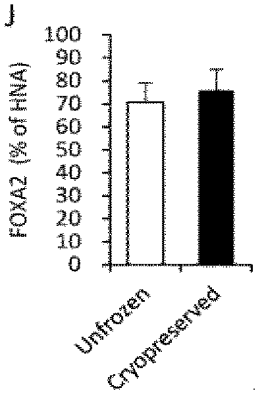
[図8I]

I



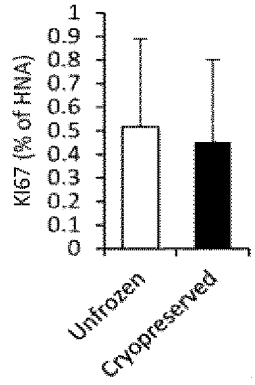
[図8J]

J

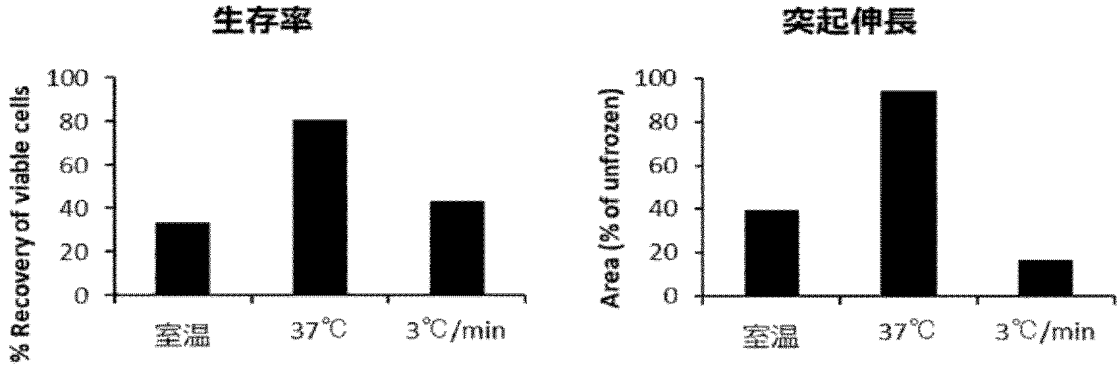


[図8K]

K

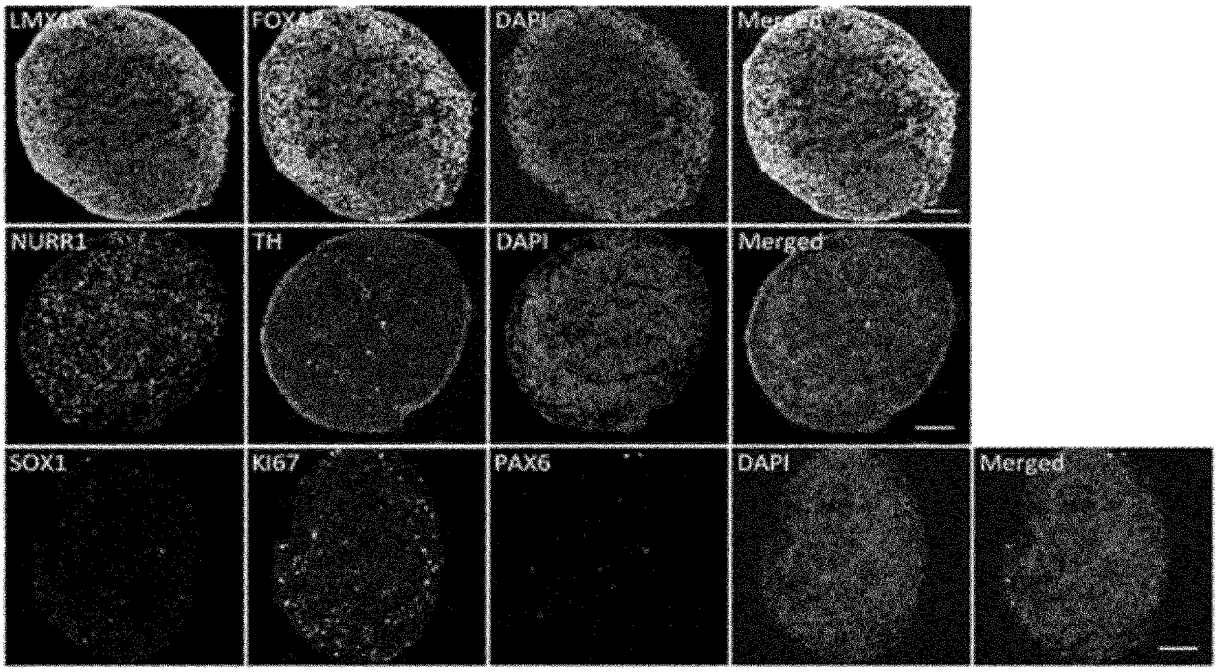


[図9]

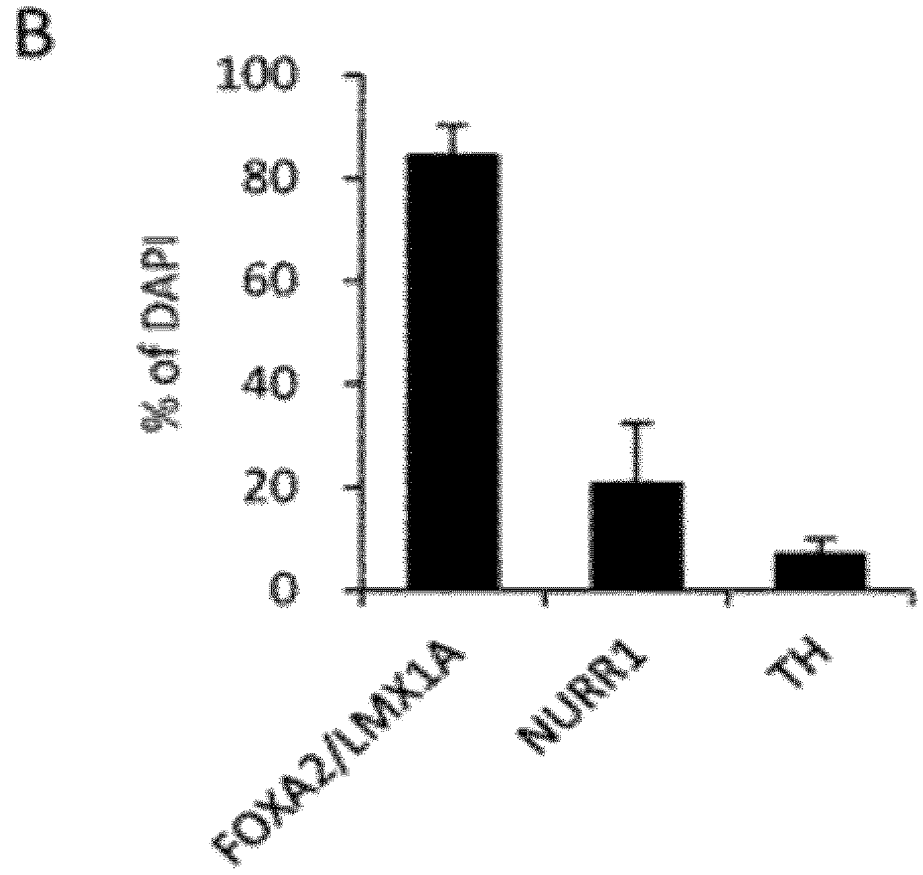


[図10A]

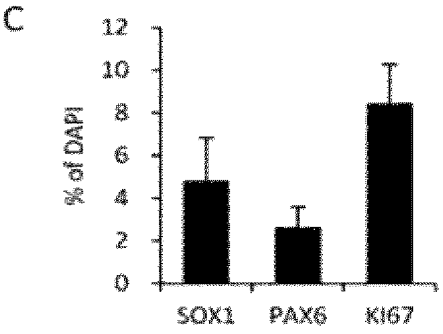
A



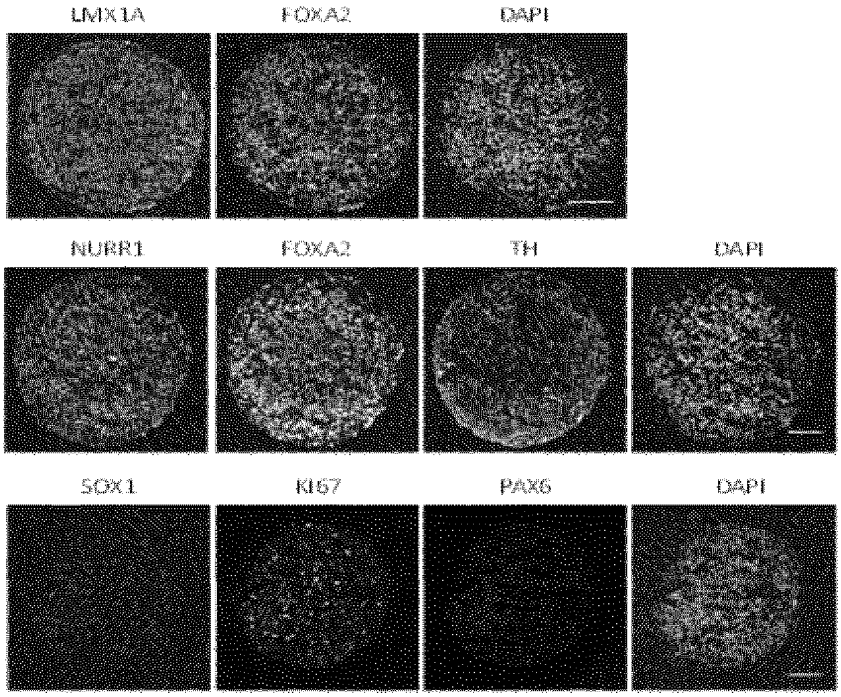
[図10B]


B
100
80
60
40
20
0
% of DAPI
FOXA2/LMX1A
NURR1
TH

[図10C]


C
12
10
8
6
4
2
0
% of DAPI
SOX1
PAX6
KI67

[図11]



INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2020/043275

A. CLASSIFICATION OF SUBJECT MATTER
C12N 5/10(2006.01)i; A61K 35/545(2015.01)i; A61P 25/16(2006.01)i; A61P 43/00(2006.01)i; C12N 1/04(2006.01)i; C12N 5/0793(2010.01)i
FI: C12N5/10; A61K35/545; A61P25/16; A61P43/00 107; A61P43/00 111; C12N5/0793; C12N1/04 ZNA
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N5/10; A61K35/545; A61P25/16; A61P43/00; C12N1/04; C12N5/0793

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Published examined utility model applications of Japan	1922–1996
Published unexamined utility model applications of Japan	1971–2021
Registered utility model specifications of Japan	1996–2021
Published registered utility model applications of Japan	1994–2021

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAplus/MEDLINE/EMBASE/BIOSIS/WPIDS (STN) JSTPlus/JMEDPlus/JST7580 (JDreamIII)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP 2013-110988 A (SUMITOMO CHEMICAL CO., LTD.) 10 June 2013 (2013-06-10) claims	1-31
A	JP 2008-501320 A (ES CELL INTERNATIONAL PTE LTD.) 24 January 2008 (2008-01-24) claims	1-31
A	JP 7-500597 A (CELLPRO, INCORPORATED) 19 January 1995 (1995-01-19) claims	1-31

☐ Further documents are listed in the continuation of Box C.
☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 January 2021 (05.01.2021)	19 January 2021 (19.01.2021)
Name and mailing address of the ISA/ Japan Patent Office 3-4-3, Kasumigaseki, Chiyoda-ku, Tokyo 100-8915, Japan	Authorized officer Telephone No.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/JP2020/043275

Patent Documents referred in the Report	Publication Date	Patent Family	Publication Date
JP 2013-110988 A	10 Jun. 2013	WO 2013/077424 A1 claims US 2014/0342346 A1 EP 2784154 A1	
JP 2008-501320 A	24 Jan. 2008	WO 2005/118785 A1 claims US 2008/0057040 A1 EP 1765986 A1	
JP 7-500597 A	19 Jan. 1995	WO 1993/007745 A1 claims EP 609379 A1	

国際調査報告

国際出願番号
PCT/JP2020/043275

A. 発明の属する分野の分類（国際特許分類（ＩＰＣ））

C12N 5/10(2006.01)i; A61K 35/545(2015.01)i; A61P 25/16(2006.01)i; A61P 43/00(2006.01)i;
C12N 1/04(2006.01)i; C12N 5/0793(2010.01)i
FI: C12N5/10; A61K35/545; A61P25/16; A61P43/00 107; A61P43/00 111; C12N5/0793; C12N1/04 ZNA

B. 調査を行った分野

調査を行った最小限資料（国際特許分類（ＩＰＣ））

C12N5/10; A61K35/545; A61P25/16; A61P43/00; C12N1/04; C12N5/0793

最小限資料以外の資料で調査を行った分野に含まれるもの

日本国実用新案公報	１９２２－１９９６年
日本国公開実用新案公報	１９７１－２０２１年
日本国実用新案登録公報	１９９６－２０２１年
日本国登録実用新案公報	１９９４－２０２１年

国際調査で使用した電子データベース（データベースの名称、調査に使用した用語）

CAplus/MEDLINE/EMBASE/BIOSIS/WPIDS (STN) JSTPlus/JMEDPlus/JST7580 (JDreamIII)

C. 関連すると認められる文献

引用文献のカテゴリー*	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求項の番号
A	JP 2013-110988 A (住友化学株式会社) 10.06.2013 (2013-06-10) [特許請求の範囲]	1-31
A	JP 2008-501320 A (イーエス・セル・インターナショナル・プライヴェート・リミテッド) 24.01.2008 (2008-01-24) [特許請求の範囲]	1-31
A	JP 7-500597 A (セルプロ　インコーポレイテッド) 19.01.1995 (1995-01-19) 請求の範囲	1-31

☐ C欄の続きにも文献が列挙されている。

☑ パテントファミリーに関する別紙を参照。

* 引用文献のカテゴリー
"A" 特に関連のある文献ではなく、一般的技術水準を示すもの
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"O" 口頭による開示、使用、展示等に言及する文献
"P" 国際出願日前で、かつ優先権の主張の基礎となる出願の日の後に公表された文献
"T" 国際出願日又は優先日後に公表された文献であって出願と抵触するものではなく、発明の原理又は理論の理解のために引用するもの
"X" 特に関連のある文献であって、当該文献のみで発明の新規性又は進歩性がないと考えられるもの
"Y" 特に関連のある文献であって、当該文献と他の１以上の文献との、当業者にとって自明である組合せによって進歩性がないと考えられるもの
"&" 同一パテントファミリー文献

国際調査を完了した日	国際調査報告の発送日
05.01.2021	19.01.2021

名称及びあて先

日本国特許庁(ISA/JP)
〒100-8915
日本国
東京都千代田区霞が関三丁目４番３号

権限のある職員（特許庁審査官）

坂井田　京 4N 1150

電話番号 03-3581-1101 内線 3488

国際調査報告

国際出願番号
PCT/JP2020/043275

第Ⅰ欄　　ヌクレオチド又はアミノ酸配列（第１ページの１．ｃの続き）

1. この国際出願で開示されたヌクレオチド又はアミノ酸配列に関して、以下の配列表に基づき国際調査を行った。

a. ☑ 出願時における国際出願の一部を構成する配列表
- ☑ 附属書C/ST.25テキストファイル形式
- ☐ 紙形式又はイメージファイル形式

b. ☐ 国際出願とともに、ＰＣＴ規則13の3.1(a)に基づき国際調査のためにのみ提出された、附属書C/ST.25テキストファイル形式の配列表

c. ☐ 国際出願日後に、国際調査のためにのみ提出された配列表
- ☐ 附属書C/ST.25テキストファイル形式(ＰＣＴ規則13の3.1(a))
- ☐ 紙形式又はイメージファイル形式（ＰＣＴ規則13の3.1(b)及びＰＣＴ実施細則第713号）

2. ☐ さらに、複数の版の配列表又は配列表の写しが提出され、変更後の配列表又は追加の写しに記載された情報が、出願時における配列表と同一である旨、又は出願時における国際出願の開示の範囲を超えない旨の陳述書の提出があった。

3. 補足意見:

国際調査報告
パテントファミリーに関する情報

国際出願番号
PCT/JP2020/043275

引用文献	公表日	パテントファミリー文献	公表日
JP 2013-110988 A	10.06.2013	WO 2013/077424 A1 Claims US 2014/0342346 A1 EP 2784154 A1	
JP 2008-501320 A	24.01.2008	WO 2005/118785 A1 Claims US 2008/0057040 A1 EP 1765986 A1	
JP 7-500597 A	19.01.1995	WO 1993/007745 A1 Claims EP 609379 A1	

METHOD FOR FREEZING NEURAL CELLS

TECHNICAL FIELD

[0001] The present application relates to a method for freezing cell aggregates including neural cells.

BACKGROUND

[0002] Transplantation of dopamine-producing (DA) neurons has been considered as a promising therapeutic method for Parkinson's disease (PD) based on reports on clinical trials using fetal mesencephalic cells (Non Patent Literature 1). In addition, a method for inducing differentiation of pluripotent stem cells (PSCs) such as embryonic stem cells (ES cells or ESCs) or induced pluripotent stem cells (iPS cells or iPSCs) into dopamine-producing neurons, or progenitor cells thereof has been reported. The group of the present inventors has already reported a method for preparing dopamine-producing neurons, or dopamine-producing neuron progenitor cells from human induced pluripotent stem cells (Non Patent Literature 2). Other groups have also reported preparation of PSC-derived dopamine-producing nerves (Non Patent Literatures 3 and 4).

[0003] For drugs having cells as active ingredients, cryopreservation of a final product is an essential element for the dissemination of cell therapies (Patent Literatures 1 to 4). Unlike cell biology research, it is

preferable that cryopreserved cells, when used in clinical practice, be transplanted immediately after thawing without recovery culture. Therefore, it is important for frozen cells to maintain their engraftment capacity, function or activity, and cell viability after thawing.

[0004] It has been suggested that transplantation of solid tissue induces a stronger immune response than transplantation of cell suspension because of the remaining donor blood vessels and antigen-presenting cells (Non Patent Literature 5). On the other hand, if the immune response is suppressed by isogeneic transplantation or an immunosuppressive agent, this problem is eliminated, and transplantation of ventral midbrain (VM) tissue shows dopamine-producing nerves having a higher survival rate and behavioral recovery than transplantation of cell suspension (Non Patent Literature 6). In addition, mechanical and enzymatic dissociation processes to obtain a cell suspension can alter cell properties to cause cell injury. Therefore, in clinical applications, it is desirable that cells to be transplanted should be administered as a sphere rather than a cell suspension. However, spheres are more difficult to cryopreserve than single cells.

[0005] When cryopreserved PSC-derived dopamine-producing nerves as a single cell suspension is transplanted into rat corpus striatum, the survival ratio of TH^+ cells is reduced to about 60% compared to unfrozen cells (Non Patent

Literature 7). On the other hand, in most researches with human or rat ventral midbrain (VM) tissue cryopreserved, the dopamine-producing nerves *in vivo* have a survival rate reduced to less than 20% compared to unfrozen tissue (Non Patent Literatures 8 to 10). Therefore, there has been a need to develop a freezing method that can maintain the survival of a PSC-derived dopamine-producing neuron sphere.

[0006] In general, there are two cell cryopreservation methods (Non Patent Literatures 11 to 13). The slow cooling method of these methods is a method in which cells are frozen together with a low concentration of cryoprotectant (CPA) (such as 10% dimethyl sulfoxide (DMSO)) at about 1 °C/min (Patent Literature 5, Non Patent Literatures 14 and 15). On the other hand, the vitrification method is an ultra-fast cooling method in which cells are transferred into liquid nitrogen immediately after a high concentration of cryoprotectant is added (Patent Literature 6, Non Patent Literature 16). Since the vitrification method requires strict time control, its application to clinical cell production has been technically difficult (Non Patent Literature 17).

[0007] In the slow cooling method, on the other hand, ice formation begins first in the extracellular space, leading to the concentration of extracellular fluid. As a result, water is withdrawn from the cells by an osmotic gradient across the cell membrane. This dehydration of the cells avoids intracellular ice formation. However, if cells are

excessively dehydrated, they are injured by the concentrated intracellular fluid and by the CPA in the cryopreservation solution. Because of the need for precise control of ice formation and cell dehydration, no clinical cryopreservation method for a sphere has been established.

Citation List

Patent Literature

[0008] [Patent Literature 1]

JP2017-104061A

[Patent Literature 2]

WO2017/159862

[Patent Literature 3]

JP2015-521469A

[Patent Literature 4]

JP2008-501320A

[Patent Literature 5]

JP2011-103885A

[Patent Literature 6]

JP2013-110988A

Non Patent Literature

[0009] [Non Patent Literature 1]

Piccini et al; Nature Neuroscience, 2(12), 1137-1140, 1999

[Non Patent Literature 2]

Doi et al., Stem Cell Reports, 2(3), 337-350, 2014

[Non Patent Literature 3]

Sundberg et al., Stem Cells 31, 1548-1562, 2013

[Non Patent Literature 4]

Nolbrant et al., Nature Protocols, 12(9), 1962-1979,.2017

[Non Patent Literature 5]

Redmond et al., Neurobiology of Disease, 29(1), 103-116, 2008

[Non Patent Literature 6]

Fricker et al., PLoS ONE, 7(10), e47169, 2012

[Non Patent Literature 7]

Nolbrant et al., Nature Protocols, 12(9), 1962-1979, 2017

[Non Patent Literature 8]

Frodl et al., Brain Research, 647(2), 286-298, 1994

[Non Patent Literature 9]

Sautter et al., Journal of Neuroscience Methods, 64(2), 173-179, 1996

[Non Patent Literature 10]

Sautter et al., Experimental Neurology, 164(1), 121-129, 2000

[Non Patent Literature 11]

Chong et al., Stem Cells, 27(1), 29-39, 2009

[Non Patent Literature 12]

Smith et al., Fertility and Sterility, 94(6), 2088-2095, 2010

[Non Patent Literature 13]

Jang et al., Integrative Medicine Research, 6(1), 12-18, 2017

[Non Patent Literature 14]

Schwartz et al., Journal of Neuroscience Research, 74(6), 838-851, 2003

[Non Patent Literature 15]

Woods et al., Cryobiology, 59(2), 150-157, 2009

[Non Patent Literature 16]

Fahy and Wowk., Methods in Molecular Biology (Methods and Protocols), vol 1257. Springer, New York, NY, 2015

[Non Patent Literature 17]

Nagano et al., Biomedical Research, 28(3), 153-160, 2007

SUMMARY OF INVENTION

TECHNICAL PROBLEM

[0010] An object of the present application is to provide a method for freezing cell aggregates including neural cells.

SOLUTION TO PROBLEM

[0011] As a result of intensive studies, the present inventors have found a method for freezing a cell aggregate including neural cells and have completed the present invention. More specifically, the present invention relates to the following:

[1] A method for freezing a cell aggregate including neural cells and having a three-dimensional structure, which comprises following steps (1) and (2):

(1) contacting the cell aggregate including neural cells and having a three-dimensional structure with a preservation solution at 0°C to 30°C prior to freezing to prepare a preservation solution-soaked cell aggregate; and

(2) cooling the preservation solution-soaked cell aggregate obtained in step (1) from a temperature at least about 5°C higher than the freezing point of the preservation solution to a temperature about 5°C lower than the freezing point at an average cooling speed of 2 to 7 °C/min to freeze the cell aggregate.

[2] The method according to [1], wherein the average cooling speed in step (2) is 3 to 7 °C/min.

[3] The method according to [1] or [2], wherein the cell aggregate is contacted with the preservation solution for 15 minutes to 90 minutes, preferably 15 minutes to 60 minutes in step (1).

[4] The method according to any one of [1] to [3], wherein the preservation solution has the freezing point of from -1°C to -10°C.

[5] The method according to any one of [1] to [4], wherein the preservation solution is an aqueous solution comprising 7% to 12% of dimethyl sulfoxide and/or propylene glycol, and step (2) is a step of cooling from 0 ± 5°C to -30 ± 5°C at an average cooling speed of 2 to 5 °C/min.

[6] The method according to any one of [1] to [5], wherein the average cooling speed in step (2) is 3 to 5 °C/min.

[7] The method according to any one of [1] to [6], wherein the method further comprises a following step (3):

(3) cooling the frozen cell aggregate obtained in step (2) to -50°C or lower.

[8] The method according to any one of [1] to [7], wherein the cell aggregate including neural cells is a cell aggregate including neural cells derived from pluripotent stem cells.

[9] The method according to any one of [1] to [8], wherein the cell aggregate including neural cells comprises cells that are positive for at least one of FOXA2, TH, and NURR1.

[10] The method according to [9], wherein the cell aggregate including neural cells comprises cells positive for FOXA2 and LMX1A.

[11] The method according to [9], wherein the cell aggregate including neural cells comprises cells positive for FOXA2, TH, and NURR1.

[12] The method according to any one of [1] to [8], wherein the cell aggregate including neural cells comprises cells positive for FOXA2 and LMX1A in an amount of 40% or more of the total cells and cells positive for TH and NURR1 in an amount of 40% or less of the total cells.

[13] The method according to any one of [1] to [12], wherein the cell aggregate including neural cells

comprises a dopamine-producing neuron progenitor cell and/or a dopamine-producing neuron.

[14] The method according to any one of [1] to [13], wherein the cell aggregate includes 500 to 150000 cells.

[15] The method according to any one of [1] to [14], wherein number of cells contained in the preservation solution is 80000 to 5000000 cells/mL, and the cell aggregate has an equivalent spherical diameter of 150 to 1000 μm.

[16] The method according to any one of [1] to [15], wherein the cell aggregate and the preservation solution have volume of 0.25 mL to 2 mL.

[17] The method according to any one of [1] to [16], wherein the cell aggregate and the preservation solution are packed in a 0.5 mL to 15 mL container.

[18] A method for preserving a cell aggregate including neural cells and having a three-dimensional structure for a long-term, wherein the method comprises holding the frozen cell aggregate obtained by the method according to any one of [1] to [17] at or below -80°C.

[19] The method according to any one of [1] to [18], wherein the obtained frozen cell aggregate can be used without recovery culture after thawing.

[20] A composition for transplantation, wherein the composition comprises, as an active ingredient, the cell aggregate frozen or preserved for a long-term by the method according to any one of [1] to [19].

[21] A frozen composition for transplantation, wherein the composition comprises:

a cell aggregate including 60% or more of dopamine-producing neuron progenitor cells and dopamine-producing neurons derived from a pluripotent stem cell, having an equivalent spherical diameter of 150 μm to 1000 μm and including 500 to 150000 cells; and

a cryopreservation solution comprising 7% to 12% of dimethyl sulfoxide or propylene glycol and having the freezing point of -1°C to -10°C, and

wherein the frozen cell aggregate has following properties:

(1) about 60% or more of the total cells are viable after thawing;

(2) the cells after thawing have a neurite extension activity of 50% or more of that before freezing; and

(3) the rates of the FOXA2-positive cells, LMX1A-positive cells, NURR1-positive cells, and TH-positive cells in the cells viable after thawing are changed within ± 10% from those in the cells before freezing.

[22] The composition for transplantation according to [20] or [21], wherein number of cells in the composition is 80000 to 5000000 cells/mL, and the composition comprises cells positive for FOXA2 and LMX1A in an amount of 40% or more of the total cells and cells positive for TH and NURR1 in an amount of 40% or less of the total cells.

[23] The composition for transplantation according to any one of [20] to [22], wherein the cell aggregate has an equivalent spherical diameter of 150 to 1000 μm.

[24] The composition for transplantation according to any one of [20] to [23], wherein the composition can be used without recovery culture after thawing.

[25] The composition for transplantation according to any one of [20] to [24], wherein the composition comprises 8 to 192 cell aggregates/mL, the cell aggregate has an equivalent spherical diameter of 150 μm to 1000 μm, and number of cells per container is 80000 to 2400000.

[26] The composition for transplantation according to any one of [20] to [25], wherein the cell aggregate and the preservation solution have volume of 0.25 mL to 2 mL.

[27] The composition for transplantation according to any one of [20] to [26], wherein the composition is packed in a 0.5 mL to 15 mL container.

[28] A method for producing a composition for transplantation containing a dopamine-producing neuron progenitor cell as an active ingredient, which comprises:

freezing a cell aggregate by the method according to any one of [1] to [17], wherein number of cells in the composition is 80000 to 5000000 cells/mL, the cell aggregate comprises cells positive for FOXA2 and LMX1A in an amount of 40% or more of the total cells and cells positive for TH and NURR1 in an amount of 40% or less of

the total cells, and the cell aggregate has an equivalent spherical diameter of 150 μm to 1000 μm.

[29] The method for producing the composition for transplantation according to [28] containing an aggregate of neural cells as an active ingredient, wherein the composition comprises a population of cell aggregates which have an equivalent spherical diameter of 150 to 1000 μm, number of cells per container is 80000 to 2400000, the cell aggregate and the preservation solution have volume of 0.25 mL to 2 mL, and the composition is packed in a 0.5 mL to 15 mL container.

[30] A method for treating a disease requiring regeneration of a dopamine-producing nerve, which comprises following steps of:

(1) thawing the composition for transplantation according to any one of [20] to [27] at 30°C to 40°C, preferably at 37°C ± 3°C; and

(2) transplanting the composition for transplantation obtained in (1) into a corpus striatum region of a patient.

[31] The method according to [30], wherein the cryopreservation solution is replaced with a dosing vehicle without culture after thawing to perform step (2).

EFFECTS OF THE INVENTION

[0012] The present application provides a method for cryopreserving a cell aggregate including neural cells. The neural cells cryopreserved by the method of the present

application exhibit high cell viability and maintain functional properties.

BRIEF DESCRIPTION OF DRAWINGS

[0013] [Figure 1] A scheme showing a protocol for inducing differentiation from iPSCs into dopamine-producing neuron progenitor cells and timings for evaluations. Abbreviations in the figure indicate components added to medium. LDN: LDN193189, A: A83-01, Y: Y-27632, Pur: Purmorphamine, CHIR: CHIR99021, AA: ascorbic acid.

[0014] [Figure 2A] The effect of cryopreservation solutions on iPSC-derived dopamine-producing neuron progenitor cells. Recovery of viable cells after thawing from unsorted cells that were immersed in the cryopreservation solutions shown in Table 1 for 15 minutes and then cryopreserved at 0.5 °C/min (n = 4).

[Figure 2B] The effect of cryopreservation solutions on iPSC-derived dopamine-producing neuron progenitor cells. Neurite extension of spheres derived from the unsorted cells that were immersed in the cryopreservation solutions shown in Table 1 for 15 minutes and then cryopreserved at 0.5 °C/min (n = 4).

[Figure 2C] The effect of a cryopreservation solutions on iPSC-derived dopamine-producing neuron progenitor cells. Shown is immunostained images obtained by staining, with an early neuronal marker (PSA-NCAM), neurites of spheres derived from unsorted cells that were immersed in the

cryopreservation solutions shown in Table 1 for 15 minutes and then cryopreserved at 0.5 °C/min. The scale bar indicates 1 mm.

[0015] [Figure 3] Time-temperature curves for a sample (straight line), a freezing chamber (dashed line), and a program (dotted line). Bambanker hRM was used as the sample. The lower graph shows an enlargement of the temperature change caused by latent heat release in the upper graph.

[0016] [Figure 4A] The effect of cryopreservation programs on iPSC-derived dopamine-producing neuron progenitor cells. Recovery of viable cells after thawing from unsorted cells that were immersed in Bambanker hRM for 15 minutes and then cryopreserved according to different freezing programs.

[Figure 4B] The effect of a cryopreservation programs on iPSC-derived dopamine-producing neuron progenitor cells. Neurite extension of spheres derived from the unsorted cells that were immersed in Bambanker hRM for 15 minutes and then cryopreserved according to different freezing programs.

[0017] [Figure 5A] The effect of freezing iPSC-derived dopamine-producing neuron progenitor cells after prolonged exposure to the cryopreservation solutions. Recovery of viable cells after thawing in unsorted cells that were exposed to Bambanker hRM for 60 minutes and then cryopreserved in the different freezing programs.

[Figure 5B] The effect of freezing iPSC-derived dopamine-producing neuron progenitor cells after prolonged exposure to the cryopreservation solutions. Neurite extension of spheres derived from the unsorted cells that were exposed to Bambanker hRM for 60 minutes and then cryopreserved in the different freezing programs.

[0018] [Figure 6A] *In vitro* characteristics of the cryopreserved sphere. Recovery of viable cells after thawing.

[Figure 6B] *In vitro* characteristics of the cryopreserved sphere. Neurite extension of the sphere.

[Figure 6C] *In vitro* characteristics of the cryopreserved sphere. Immunostaining of the sphere at day 35 and day 7 after thawing. The left panels show immunostaining images for FOXA2/DAPI, the center panels show immunostaining images for NURR1/TH, and the right panels show immunostaining images for SOX1/KI67/PAX6/DAPI. The scale bar indicates 100 μm.

[Figure 6D] *In vitro* characteristics of the cryopreserved sphere. Percentages of $FOXA2^+$ cells, $NURR1^+$ cells, and TH^+ cells to total cells at day 35 and day 7 after thawing.

[Figure 6E] *In vitro* characteristics of the cryopreserved sphere. Percentages of $SOX1^+$ cells, $PAX6^+$ cells, and $KI67^+$ cells to total cells at day 35 and day 7 after thawing.

[Figure 6F] *In vitro* characteristics of the cryopreserved sphere. The gene expression of the spheres relative to GAPDH measured by quantitative RT-PCR. The expression level of undifferentiated cells (day 0) was set to 1.

[Figure 6G] *In vitro* characteristics of the cryopreserved sphere. The gene expression of the spheres relative to GAPDH measured by quantitative RT-PCR. The expression level of undifferentiated cells (day 0) was set to 1.

[Figure 6H] *In vitro* characteristics of the cryopreserved sphere. A principal component analysis of the microarray data. Temporal changes in gene expressions of unfrozen cells (circle) and frozen cells (triangle) are shown.

[Figure 6I] *In vitro* characteristics of the cryopreserved sphere. Scatter plots of the microarray data from unfrozen cells and frozen cells in the same lot at day 35 (X-axis) or day 7 after thawing (Y-axis) are shown. A solid black circle indicates a gene showing a signal intensity of 50 or more in either one of the samples, and a white circle indicates a gene showing a signal intensity of 50 or less in both samples.

[Figure 6J] *In vitro* characteristics of the cryopreserved sphere. Scatter plots of microarray data from unfrozen cells in different lots at day 35 are shown. A solid black circle indicates a gene showing a signal

intensity of 50 or more in either one of the samples, and a white circle indicates a gene showing a signal intensity of 50 or less in both samples.

[Figure 6K] *In vitro* characteristics of the cryopreserved sphere. Immunostaining images for TUBB3, TH, and DAPI in iPSC-derived dopamine-producing neurons at day 28 + 21 after thawing. The scale bar indicates 50 μm.

[Figure 6L] *In vitro* characteristics of the cryopreserved sphere. A representative induced action potential of iPSC-derived dopamine-producing neurons at day 28 + 21 after thawing.

[Figure 6M] *In vitro* characteristics of the cryopreserved sphere. Results of dopamine release induced by high potassium stimulation at day 56 or day 28 + 28 after thawing.

[0019] [Figure 7A] Temporal change in the expression of a dopamine-producing neuron progenitor cell marker after thawing. Percentage of $FOXA2^+$ cells to total cells at days 28, 29, 31, and 35 and days 0, 1, 3, and 7 after thawing.

[Figure 7B] Temporal change in the expression of a dopamine-producing neuron progenitor cell marker after thawing. Percentage of $NURR1^+$ cells to total cells at days 28, 29, 31, and 35 and days 0, 1, 3, and 7 after thawing.

[Figure 7C] Temporal change in the expression of a dopamine-producing neuron progenitor cell marker after thawing. The gene expression for TH of the sphere relative

to GAPDH measured by quantitative RT-PCR. The expression level of undifferentiated cells (day 0) was set to 1.

[0020] [Figure 8A] The graft survival rate and function of a cryopreserved sphere. Methamphetamine-induced rotational movements of rats having grafts. Data are presented as the mean ± SEM (n = 6 to 8). Two-way ANOVA with Tukey's multiple comparison test was performed, and significance levels are indicated by **p <0.01, ****p <0.001 relative to a solvent-administered group.

[Figure 8B] The graft survival rate and function of a cryopreserved sphere. Immunostaining images for HNA of representative grafts derived from unfrozen cells (upper panel) and frozen cells (lower panel).

[Figure 8C] The graft survival rate and function of a cryopreserved sphere. Number of survived HNA^+ cells in grafts.

[Figure 8D] The graft survival rate and function of a cryopreserved sphere. A DAB-staining image for TH of a representative graft derived from unfrozen cells.

[Figure 8E] The graft survival rate and function of a cryopreserved sphere. A magnified image within the frame of Figure 8D. The right panel is a magnified image within the frame of the left panel. The scale bar indicates 200 μm.

[Figure 8F] The graft survival rate and function of a cryopreserved sphere. A DAB-staining image for TH of a representative graft derived from frozen cells.

[Figure 8G] The graft survival rate and function of a cryopreserved sphere. A magnified image within the frame of Figure 8F. The right panel is a magnified image within the frame of the left panel. The scale bar indicates 200 μm.

[Figure 8H] The graft survival rate and function of a cryopreserved sphere. Number of survived TH^+ cells in grafts.

[Figure 8I] The graft survival rate and function of a cryopreserved sphere. Immunostaining images for FOXA2, TH, and HNA (upper), and KI67 and HNA (lower) of grafts derived from frozen cells. The scale bar indicates 50 μm.

[Figure 8J] The graft survival rate and function of a cryopreserved sphere. Percentages of $FOXA2^+$ cells relative to HNA^+ cells.

[Figure 8K] The graft survival rate and function of a cryopreserved sphere. Percentages of $KI67^+$ cells relative to HNA^+ cells.

[Figure 9] The survival rate and neurite extension activity of cryopreserved spheres thawed under different conditions.

[Figure 10A] The expression of markers in a sphere derived from cells not sorted with anti-CORIN antibodies. Immunostaining images for LMX1A, FOXA2, and DAPI (upper), NURR1, TH, and DAPI (center), and SOX1, KI67, PAX6, and DAPI (lower) of the sphere. The scale bar indicates 100 μm.

[Figure 10B] The expression of markers in a sphere derived from cells not sorted with anti-CORIN antibodies. Percentages of $FOXA2^+/LMX1A^+$ cells, $NURR1^+$ cells, and TH^+ cells relative to total cells.

[Figure 10C] The expression of markers in a sphere derived from cells not sorted with anti-CORIN antibodies. Percentages of $SOX1^+$ cells, $PAX6^+$ cells, and $KI67^+$ cells relative to total cells.

[Figure 11] The expression of markers in a sphere immediately before freezing (day 28) in the same lot as the sphere shown in Figure 6C. Immunostaining images for LMX1A, FOXA2, and DAPI (upper), NURR1, FOXA2, TH, and DAPI (center), and SOX1, KI67, PAX6, and DAPI (upper) of spheres. The scale bar indicates 100 μm.

DESCRIPTION OF EMBODIMENTS

[0021] In the present specification and claims, when a numerical value is accompanied by the term "about," it is intended to include a range of ± 10% of the value. For example, "about 20" shall include "18 to 22". A numerical range includes all numerical values between the two endpoints and the numerical values at both endpoints. The term "about" for a range applies to both endpoints of the range. Thus, for example, "about 20 to 30" shall include "18 to 33".

[0022][Neural cells]

The present application provides a method for freezing a cell aggregate including neural cells and having a three-dimensional structure.

[0023] The neural cells include neuronal cells or neurons, progenitor cells of the neurons, i.e., neural progenitor cells or neural precursor cells.

The neural cells may be neural cells derived from any site such as neural cells of the central nervous system, or neural cells of the peripheral nervous system such as somatic neural cells of motor nerves or sensory systems or neural cells of autonomic nerves; examples of the neural cells include nerves (neurons), neural crest-derived cells, glia cells such as oligodendrocytes or astrocytes, and stem cells or progenitor cells thereof. Examples of the neural cell include a cell expressing a neural cell marker. Examples of the neural cell marker include, but are not limited to, NCAM, βIII-Tubulin (TUJ1), tyrosine hydroxylase (TH), serotonin, nestin, MAP2, MAP2AB, NEUN, GABA, glutamate, CHAT, SOX1, BF1, EMX1, VGLUT1, PAX, NKX, GSH, Telencephalin, GLUR1, CAMKII, CTIP2, TBR1, Reelin, TBR1, BRN2, OTX2, LMX1A, LMX1B, EN1, NURR1, PITX3, DAT, GIRK2, and TH. The neural cell can be identified by the expression of one or more neural cell markers. In the present specification, examples of the neural cell include a cell expressing one or more, two or more, or three or more of the above neural cell markers.

[0024] Neural cells of the central nervous system can be classified according to differences in sites where the neural cells nervous systems are present. That is, examples of the neural cells of the central nervous system include neurons derived from prosencephalon, telencephalon, diencephalon, cerebral, hypothalamus, mesencephalon, metencephalon, mesencephalon-metencephalon boundary region, cerebellum, retina, pituitary gland, or spinal cord, and progenitor cells thereof.

[0025] The neurons derived from prosencephalon are neurons that are present in prosencephalon tissue (i.e., telencephalon, cerebral, hippocampus or choroid plexus, diencephalon, hypothalamus, etc.). The neurons of prosencephalon can be identified by the expression of a prosencephalon neuron marker. Examples of the prosencephalon neuron marker include OTX1 (prosencephalon), BF1 (also referred to as FOXG1) or SIX3 (also serving as a marker for telencephalon or cerebral). In the present specification, examples of the neural cell include a cell expressing one or more, two or more, or three or more of the above prosencephalon neuron markers or the markers for telencephalon or cerebral.

[0026] Examples of the neuron derived from cerebral include dorsal cells (e.g., cerebral cortex cells, Cajal-Retzius cells, hippocampus neurons) and ventral cells (e.g., basal ganglia). Examples of the ventral cerebral neuron marker include basal ganglia neuron markers (e.g., GSH2, MASH1,

NKX2.1, NOZ1). Examples of the dorsal cerebral neuron marker include cerebral cortex neuron markers (e.g., PAX6, EMX1, TBR1). In the present specification, examples of the neural cell include a cell expressing one or more, two or more, or three or more of the above cerebral neuron markers, the basal ganglia neuron markers or the cerebral cortex neuron markers.

[0027] Examples of the neural cell derived from mesencephalon include neural progenitor cells derived from ventral mesencephalon, dopamine-producing neurons (also referred to as dopaminergic neurons) and dopamine-producing neuron progenitor cells (also referred to as dopaminergic neuron progenitor cells or dopaminergic progenitors). Examples of a mesencephalon-derived neural cell marker include FOXA2, EN2, and TUJ1. Examples of a FOXA2-positive and TUJ1-positive neural cell include dopamine-producing neuron progenitor cells and dopamine-producing neurons. The dopamine-producing neurons can be identified by being FOXA2-positive, NURR1-positive, and TH-positive.

[0028] The dopamine-producing neuron progenitor cells can be identified by being FOXA2-positive and LMX1A-positive. The dopamine-producing neuron progenitor cells more preferably contain cells that are positive for one or more of OTX2, LMX1A, LMX1B, CORIN, SHH, AADC, βIII-Tubulin, EN1, NURR1, PITX3, DAT, GIRK2, and TH. In the present specification, a cell aggregate including dopamine-

producing neuron progenitor cells, unless otherwise specified, may comprise dopamine-producing neurons or dopaminergic neurons.

[0029] In the present specification, examples of the neural cell include a cell expressing one or more, two or more, or three or more of the mesencephalon-derived neural cell markers, the dopamine-producing neuron progenitor cell markers or the dopamine-producing neuron markers.

[0030] In the present specification, examples of the dopamine-producing neuron progenitor cells include cells expressing FOXA2 and/or LMX1A (FOXA2-positive and/or LMX1A-positive), preferably cells expressing one or more, two or more, or three or more selected from the group consisting of OTX2, LMX1B, CORIN, SHH, AADC, and βIII-Tubulin in addition to FOXA2 and LMXA1.

[0031] In the present specification, examples of the dopamine-producing neurons (dopaminergic neurons) include cells expressing TH and/or NURR1 (TH-positive and/or NURR1-positive), preferably cells expressing one or more, two or more, or three or more selected from the group consisting of FOXA2, AADC, DAT, and GIRK2 in addition to TH and NURR1.

[0032] Examples of the neurons derived from the mesencephalon-metencephalon boundary region include neurons that are present in the cerebellum, cerebellar plate tissue, ventricular zone, rhombic lip, etc. Examples of a mesencephalon-metencephalon boundary region

marker include EN2 (mesencephalon), GBX2 (metencephalon), N-Cadherin (neural progenitor cells in the mesencephalon-metencephalon boundary region). Examples of a cerebellar neural progenitor cell marker include KIRREL2, PTF1A, and SOX2, which are GABAergic progenitor cell markers, and ATOH1 and BARHL1, which are cerebellar granule cell progenitor cell markers. In the present specification, examples of the neural cell include a cell expressing one or more, two or more, or three or more of the above mesencephalon-metencephalon boundary region markers, the cerebellar neural progenitor cell markers, the GABAergic progenitor cell markers, or the cerebellar granule cell progenitor cell markers.

Examples of a neural cell derived from a retina include photoreceptor cells, photoreceptor progenitor cells, retinal pigment epithelium cells, and cornea cells. [0033] The neural cells can also be classified according to differences in neurotransmitters produced (secreted); examples thereof include dopamine-producing neurons, dopamine-producing neuron progenitor cells, GABAergic neurons, GABAergic progenitor cells, cholinergic neurons, cholinergic progenitor cells, serotonergic neurons, serotonergic progenitor cells, glutamatergic neurons, glutamatergic progenitor cells, noradrenergic neurons, noradrenergic progenitor cells, adrenergic neurons, and adrenergic progenitor cells.

[0034] Examples of the neural cells of motor nerves or sensory systems include cholinergic neurons and progenitor cells thereof.

Examples of the neural cells of autonomic nerves include cholinergic neurons, adrenergic neurons, and progenitor cells thereof.

[0035] Preferred examples of the neural cells in the present specification include dopamine-producing neurons (dopaminergic neurons), and dopamine-producing neuron progenitor cells (dopaminergic neuron progenitor cells).

[0036] Neural cells derived from a living body are cells isolated from a mammal such as humans, and examples of the cells isolated from human brain tissue include cells contained in the fetal mesencephalon tissue as described in Nature Neuroscience, 2, 1137 (1999) or N. Engl. J. Med.; 344: 710-9 (2001).

[0037] The neural cells may also be cells obtained by inducing differentiation from pluripotent stem cells such as embryonic stem cells (ES cells) and iPS cells. Examples of a method for inducing differentiation from pluripotent stem cells into neural cells include the methods described in Non Patent Literatures 3 and 4 and WO2015/034012 (dopamine-producing neuron progenitor cells), WO2009/148170 (cerebral neural cells and the like), WO2013/065763, WO2016/013669 or WO2017/126551 (hypophysial or subthalamic neural cells), WO2016/039317 (cerebellar neural cells), WO2015/076388 (telencephalic neural cells),

Numasawa-Kuroiwa, Y et al., Stem Cell Reports, 2: 648-661 (2014) (neural progenitor cells), Qiu, L et al., Stem Cells Transl Med. 6 (9): 1803-1814 (2017) (dopamine-producing neuron progenitor cells).

[0038] The neural cells may be cells obtained by inducing differentiation from multipotent stem cells such as mesenchymal stem cells (MSCs). Examples of a method for inducing differentiation from mesenchymal stem cells into neural cells include the method described in J Chem Neuroanat. 96: 126-133 (2019).

[0039]

[Pluripotent stem cell]

A pluripotent stem cell refers to a stem cell that has both pluripotency that enables differentiation into almost all cells existing in a living body and proliferative capacity. Pluripotent stem cells can be derived from fertilized ova, cloned embryos, germ stem cells, interstitial stem cells, or somatic cells. Examples of the pluripotent stem cell include, but are not particularly limited to, embryonic stem (ES) cells, nuclear transfer embryonic stem (ntES) cells derived from cloned embryos, spermatogonial stem cells (GS cells), embryonic germ cells (EG cells), induced pluripotent stem (iPS) cells, pluripotent stem cells derived from cultured fibroblasts and bone marrow stem cells (Muse cells). The pluripotent stem cells may be ES cells, ntES cells, or iPS cells. The pluripotent stem cells may be iPS cells in

view of ethical considerations. Note that the embryonic stem cells are established from embryos within 14 days of fertilization.

[0040] Embryonic stem cells were first established in 1981 and have been applied to the production of knockout mice since 1989. Human embryonic stem cells were established in 1998 and are being used in regenerative medicine. Embryonic stem cells can be produced by culturing an inner cell mass on feeder cells or in medium containing leukemia inhibitory factor (LIF). Methods for producing embryonic stem cells are described, for example, in WO96/22362, WO02/101057, US5,843,780, US6,200,806, US6,280,718, etc. Embryonic stem cells can be obtained from a given institution or purchased commercially. For example, human embryonic stem cells KhES-1, KhES-2, and KhES-3 are available from Institute for Frontier Life and Medical Sciences, Kyoto University. Human embryonic stem cell line Rx::GFP (derived from the KhES-1 line) is available from Institute of Physical and Chemical Research. Mouse embryonic stem cell lines, EB5 and D3, are available from Institute of Physical and Chemical Research and ATCC, respectively.

[0041] A nuclear transfer embryonic stem cell (ntES cell), which is one type of embryonic stem cell, can be established from a cloned embryo created by transferring the nucleus of a somatic cell into an ovum from which the nucleus has been removed.

[0042] EG cells can be produced by culturing primordial germ cells in medium containing mSCF, LIF, and bFGF (Cell, 70: 841-847, 1992).

[0043] The term "induced pluripotent stem cell" as used herein refers to a cell obtained by reprogramming a somatic cell to have pluripotency induced using a known method. Specific examples thereof include cells obtained by reprogramming differentiated somatic cells such as fibroblasts or peripheral blood mononuclear cells by expression of any combination of a plurality of genes selected from a reprogramming gene group including OCT3/4, SOX2, KLF4, MYC (c-MYC, N-MYC, L-MYC), GLIS1, NANOG, SALL4, LIN28, ESRRB to induce pluripotency. Preferred examples of the combination of reprogramming factors include (1) OCT3/4, SOX2, KLF4, and MYC (c-MYC or L-MYC), (2) OCT3/4, SOX2, KLF4, LIN28, and L-MYC (Stem Cells, 2013; 31: 458 to 466), and (3) OCT3/4, SOX2, NANOG, and LIN28 (Science 2007; 318: 1917 to 1920).

[0044] Induced pluripotent stem cells were established with mouse cells by Yamanaka et al. in 2006 (Cell, 2006, 126 (4), pp. 663 to 676). Induced pluripotent stem cells were also established with human fibroblasts in 2007 and have pluripotency and replication competence similar to embryonic stem cells (Cell, 2007, 131 (5), pp. 861 to 872; Science, 2007, 318 (5858), pp. 1917 to 1920; Nat. Biotechnol., 2008, 26 (1), pp. 101 to 106).

[0045] Induced pluripotent stem cells can also be produced by a method of deriving induced pluripotent stem cells from somatic cells through the addition of a compound or the like, in addition to a method of producing induced pluripotent stem cells by reprogramming directly through gene expression (Science, 2013, 341, pp. 651 to 654).

[0046] It is also possible to obtain an established induced pluripotent stem cell line; for example, human induced pluripotent stem cell lines such as 201B7 cells, 201B7-Ff cells, 253G1 cells, 253G4 cells, 1201C1 cells, 1205D1 cells, 1210B2 cells, 1231A3 cells, etc. established at Kyoto University are available from Kyoto University. For example, Ff-I01 cells, Ff-I01s04 cells, QHJ-I01 and Ff-I14 cells established at Kyoto University are available from Kyoto University as the established induced pluripotent stem cell line.

[0047] Examples of somatic cells used in the production of induced pluripotent stem cells include, but are not particularly limited, tissue-derived fibroblasts, erythroid cells (e.g., peripheral blood mononuclear cells (PBMCs), T cells), hepatocytes, pancreatic cells, enterocytes, and smooth muscle myocytes.

[0048] For reprogramming through expression of several genes in the production of induced pluripotent stem cells, the means for expressing the genes is not particularly limited. Examples of the above-mentioned means include infection with a viral vector (e.g., retroviral vectors,

lentiviral vectors, Sendai viral vectors, adenoviral vectors, or adeno-associated virus vectors), gene transfer (e.g., calcium phosphate transfection, lipofection, RetroNectin method, or electroporation) using a plasmid vector (e.g., plasmid vectors or episomal vectors), gene transfer (e.g., calcium phosphate transfection, lipofection, or electroporation) using an RNA vector, and direct injection (e.g., needle-based method, lipofection, or electroporation) of a protein.

[0049] Induced pluripotent stem cells can be produced in the presence of feeder cells or in the absence of feeder cells (feeder-free). In the production of induced pluripotent stem cells in the presence of feeder cells, the induced pluripotent stem cells can be produced in the presence of an undifferentiated-state maintenance factor by a known method. Medium used to produce induced pluripotent stem cells in the absence of feeder cells is not particularly limited and can be any known maintenance medium for embryonic stem cells and/or induced pluripotent stem cells, or any medium for establishing induced pluripotent stem cells in a feeder-free manner. Examples of the medium for establishing induced pluripotent stem cells in a feeder-free manner include feeder-free media such as Essential 8 medium (E8 medium), Essential 6 medium, TeSR medium, mTeSR medium, mTeSR-E8 medium, Stabilized Essential 8 medium, and StemFit medium. In the production of induced pluripotent stem cells, for example, four

factors, OCT3/4, SOX2, KLF4, and MYC (L-MYC or C-MYC) can be transferred into somatic cells in a feeder-free manner using Sendai viral vectors to prepare the induced pluripotent stem cells.

[0050] Pluripotent stem cells used in the present invention are mammalian pluripotent stem cells, preferably rodent (e.g., mouse or rat) or primate (e.g., human or monkey) pluripotent stem cells, more preferably human or mouse pluripotent stem cells, even more preferably human induced pluripotent stem cells (iPS cells) or human embryonic stem cells (ES cells).

[0051]

[Cell aggregate]

The cell aggregate "having a three-dimensional structure" herein refers to a cell aggregate (cell aggregate or sphere) which is a three-dimensional cell population formed by cultured cells adhering to each other through, for example, a suspension culture or a 3D culture. A cell aggregate of neural cells is also referred to as a neurosphere. The shape of the cell aggregate is not particularly limited and may be spherical or non-spherical. The cell aggregate in the present specification is preferably a cell aggregate having a three-dimensional shape similar to a sphere. The three-dimensional shape similar to a sphere is a shape having a three-dimensional structure and shows, for example, a circular shape or an

elliptical shape when projected onto a two-dimensional plane.

[0052] The cell aggregate including neural cells and having a three-dimensional structure has no particular restrictions on its size but usually has an equivalent spherical diameter of 150 μm to 1000 μm, and for example, 200 μm to 800 μm or 300 μm to 500 μm in one embodiment. The cell aggregate including neural cells and having a three dimensional structure usually includes 500 to 150000 cells, and in one embodiment, for example, 1000 to 100000 cells, 1000 to 70000 cells, or 3000 to 30000 cells.

[0053] The cell aggregate including neural cells may comprise other cells together with the neural cells. Examples of such a cell aggregate include a cell aggregate including 60% or more, 70% or more, 80% or more, and more preferably 90% or more of neural cells.

[0054] In one embodiment, the cell aggregate including neural cells may comprise 60% or more, 70% or more, or 80% or more of dopamine-producing neuron progenitor cells and/or dopamine-producing neurons. That is, the cell aggregate including neural cells may comprise neural cells expressing one or more markers selected from FOXA2, LMX1A, LMX1B, NURR1, and TH in an amount of 60% or more, 70% or more, or 80% or more.

[0055] In one embodiment, the cell aggregate including neural cells comprises 40% or more, 60% or more, 70% or

more, 80% or more, 85% or more, or 90% or more of dopamine-producing neuron progenitor cells.

[0056] In one embodiment, the cell aggregate including neural cells comprises cells expressing one or more, two or more, or three or more markers for dopamine-producing neuron progenitor cells in an amount of 40% or more, 60% or more, 70% or more, 80% or more, 85% or more, or 90% or more.

[0057] In one embodiment, the cell aggregate including neural cells comprises cells positive for FOXA2 and LMX1A in an amount of 40% or more, 60% or more, 70% or more, 80% or more, 85% or more, or 90% or more. In one embodiment, the cell aggregate further comprises cells positive for TH and NURR1 in an amount of 40% or less.

[0058] In one embodiment, the cell aggregate including neural cells may comprise cells positive for FOXA2, TH, and NURR1 in an amount of 0% or more, 10% or more, or 20% or more.

[0059] In one embodiment, the cell aggregate including dopamine-producing neuron progenitor cells may comprise NURR1-positive cells in an amount of 60% or less, 50% or less, 40% or less, 5 to 50%, 5 to 40%, or 5 to 20%.

[0060] In one embodiment, the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons may comprise TH-positive cells in an amount of 30% or less, 20% or less, 1 to 30%, 5 to 30%, 1 to 20%, 5 to 20%, or 5 to 15%.

[0061] In one embodiment, the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons may comprise KI67-positive cells in an amount of 30% or less, 1 to 25%, 1 to 20%, or 5 to 20%.

[0062] In one embodiment, the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons may comprise SOX1-positive cells in an amount of 20% or less, 10% or less, 5% or less, or 1% or less.

[0063] In one embodiment, the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons may comprise PAX6-positive cells in an amount of 5% or less, 2% or less, 1% or less, or 0.5% or less.

[0064] In one embodiment, the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons further comprises cells positive for TH and NURR1 in an amount of 20% or less, specifically 1% to 20%, more specifically 5% to 15%.

[0065] In one embodiment, the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons comprises cells positive for FOXA2 and LMX1A in an amount of 50% or more, preferably 60% or more, 70% or more, or 80% or more, and cells positive for TH and NURR1 in an amount of 20% or less, 1% to 20%, more specifically 5% to 15%.

[0066] In one embodiment, in the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons, SOX1-positive cells are 10% or less, preferably 7% or less, more preferably 3% or less, and PAX6-positive cells are 5% or less, preferably 4% or less, more preferably 2% or less.

[0067] In one embodiment, the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons comprises cells positive for FOXA2 and LMX1A in an amount of 60% or more and cells positive for TH and NURR1 in an amount of 1% to 20%, and SOX1-positive cells are 10% or less, preferably 7% or less, more preferably 3% or less, and PAX6-positive cells are 5% or less, preferably 4% or less, more preferably 2% or less.

[0068] In one embodiment, the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons comprises cells positive for FOXA2 and LMX1A in an amount of 60% or more of the total cells and may comprise cells positive for TH and NURR1 in an amount of 20% or less, 1 to 20%, or 5 to 15% of the total cells.

[0069] In one embodiment, the above cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons is a cell aggregate having an equivalent spherical diameter of 150 μm to 1000 μm.

[0070] In one embodiment, the above cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons comprises cells positive for FOXA2 and LMX1A in an amount of 60% or more and cells positive for NURR1 and TH in an amount of 1% to 20% and has an equivalent spherical diameter of 150 μm to 1000 μm.
[0071]
[Freezing method]

The method of the present application comprises the step of (1) contacting a cell aggregate including neural cells and having a three-dimensional structure with a preservation solution at 0°C to 30°C prior to freezing to prepare a preservation solution-soaked cell aggregate.
[0072] In the present application, a cryopreservation solution (preservation solution) refers to an aqueous solution comprising a cryoprotectant. The cryoprotectant refers to a substance that have a high affinity with water molecules and are highly effective in inhibiting the growth of ice crystals in the cryopreservation solution. Cryoprotectants include, for example, dimethyl sulfoxide (DMSO), ethylene glycol (EG), propylene glycol (PG), 1,2-propanediol (1,2-PD), 1, 3-propanediol (1,3-PD), butylene glycol (BG), isoprene glycol (IPG), dipropylene glycol (DPG), and/or glycerin. In the present application, the cryoprotectant is preferably dimethyl sulfoxide and/or propylene glycol. A concentration of the cryoprotectant in the cryopreservation solution is usually 7 to 12%,

preferably about 10%, when dimethyl sulfoxide and/or propylene glycol is used as the cryoprotectant.

[0073] As the aqueous solution, for example, physiological saline, a buffered solution such as PBS, EBSS, or HBSS, a medium for culturing cells or tissues such as DMEM, GMEM, or RPMI, serum, a serum substitute, or a mixture thereof can be used.

[0074] As the cryopreservation solution, a commercially available cryopreservation solution comprising dimethyl sulfoxide (DMSO) and/or propylene glycol as a substantial component can be used. Specific examples of the cryopreservation solution include commercially available cryopreservation solutions such as STEM-CELL BANKER (SCB; ZENOAQ), STEM-CELL BANKER DMSO free (SCB DMSO free; ZENOAQ), Bambanker hRM (BBK; NIPPON Genetics), CryoStor CS5 (CS5; BioLife Solutions), CryoStor CS10 (CS10; BioLife Solutions), and Synth-a- Freeze (SaF; Thermo Fisher Scientific). For example, it is desirable to use a cryopreservation solution (such as STEM-CELL BANKER, Bambanker hRM, CryoStor CS10, or Synth-a-Freeze) comprising 7 to 12%, preferably about 10% of dimethyl sulfoxide and/or propylene glycol.

More preferably, Bambanker hRM can be used.

[0075] In the present specification, when the cell aggregate is frozen, number of cells relative to the cryopreservation solution (cell packing density) is 80000

to 5000000 cells/mL, 100000 to 4000000 cells/mL or 200000 to 2000000 cells/mL, 300000 to 1000000 cells/mL.

[0076] In the present specification, when the cell aggregate is frozen, the cell aggregate has an equivalent spherical diameter of 150 to 1000 μm, 150 μm to 600 μm, or 300 μm to 500 μm.

[0077] In the present specification, the cell aggregate and the preservation solution have volume of 0.25 mL to 2 mL, 0.5 mL to 1.5 mL, or 0.5 mL to 1 mL.

[0078] In the present specification, the cell aggregate and the preservation solution may be packed in a 0.5 mL to 15 mL, 1 mL to 5 mL, or 1 mL to 2 mL container.

[0079] The freezing point of the cryopreservation solution in the present application is not particularly limited but is usually -1°C to -10°C, preferably -3°C to -10°C, more preferably -3°C to -6°C, even more preferably about -5°C. Examples of the cryopreservation solution in the present specification include an aqueous solution comprising 7 to 12%, preferably about 10% of dimethyl sulfoxide as a substantial component and having the freezing point of -1°C to -10°C. Also, examples of the cryopreservation solution in the present specification include an aqueous solution comprising 7 to 12%, preferably about 10% of dimethyl sulfoxide as a substantial component and having the freezing point of -3°C to -6°C.

[0080] The temperature at which the cell aggregate including neural cells is contacted with the

cryopreservation solution is usually from 0°C to 30°C, preferably from 0°C to 20°C, more preferably from 0°C to 10°C, and even more preferably from 0°C to 4°C.

[0081] The period for which the cell aggregate including neural cells is contacted with the cryopreservation solution is usually 5 minutes to 240 minutes, 5 minutes to 120 minutes, preferably 5 minutes to 60 minutes, 15 minutes to 240 minutes, 15 minutes to 180 minutes, 15 minutes to 150 minutes, preferably 15 minutes to 120 minutes, 15 minutes to 90 minutes, and more preferably 15 minutes to 60 minutes.

[0082] The method of the present application also comprises the step of (2) cooling the preservation solution-soaked cell aggregate obtained in step (1) from a temperature at least about 5°C higher than the freezing point of the preservation solution to a temperature about 5°C lower than the freezing point at an average cooling speed of 2 to 7 °C/min, 2.5 to 7 °C/min, or 3 to 7 °C/min to freeze the cell aggregate.

[0083] In the method of the present application, the preservation solution-soaked cell aggregate is cooled from a temperature at least about 5°C higher than the freezing point of the preservation solution to a temperature about 5°C lower than the freezing point at an average cooling speed of 2 to 7 °C/min, 2.5 to 7 °C/min, or 3 to 7 °C/min, preferably 2 to 5.5 °C/min, 2.5 to 5.5 °C/min, or 3 to 5.5 °C/min. In a preferred embodiment, the preservation

solution-soaked cell aggregate is cooled from 0 ± 5°C to -30°C ± 5°C at an average cooling speed of 2 to 5 °C/min, 2.5 to 5 °C/min, or 3 to 5 °C/min.

[0084] The means for cooling is not particularly limited as long as the above step is achieved; a commercially available freezer can be used, and a programmed freezer (also referred to as a controlled-rate freezer) capable of controlling the temperature may be used.

[0085] In step (2), the preservation solution-soaked cell aggregate may be exposed to an electromagnetic field and/or a magnetic field. The electromagnetic field has a frequency of, for example, about 300 kHz to about 2 MHz, preferably about 500 kHz to about 1 MHz, more preferably about 600 kHz to about 1 MHz. A frequency of the magnetic field is not particularly limited but is preferably a fixed frequency. In addition, the freezing can be performed under an electrostatic field of, for example, 10 to 2000 Gauss, preferably 50 to 1000 Gauss, and more preferably 100 to 150 Gauss.

A method for achieving the above condition is not particularly limited, and for example, a programmed freezer equipped with a device for generating an electromagnetic field and a magnetic field, which is commercially available as a proton freezer (RYOHO FREEZE SYSTEMS. CO., LTD.), can be used. That is, a device having a combination of a magnetic field (a static magnetic field (SMF)), an electromagnetic wave (an alternating electric

field (AEF)), and ultra-cold air may be used. More specifically, use of an electromagnetic wave of 300 kHz to 2 Mhz can inhibit the cell aggregate from being injured by formation of ice.

[0086] The method of the present application may further comprise the step of (3) cooling the frozen cell aggregate obtained in step (2) to -50°C or lower, preferably -80°C or lower, more preferably -150°C or lower.

[0087] The means for cooling is not particularly limited, and examples thereof include deep freezers, programmed freezers, proton freezers, and use of a low-temperature medium (e.g., liquid nitrogen).

[0088] The frozen cell aggregate obtained in step (2) or (3) may be held at -80°C or lower, preferably -150°C or lower for long-term preservation.

[0089] Means for long-term preservation is not particularly limited, and examples thereof include deep freezers, programmed freezers, proton freezers, and use of a low-temperature medium (e.g., liquid nitrogen).

[0090] The frozen cell aggregate can be thawed and used as appropriate. The thawing method is not particularly limited, but it is desirable to perform the thawing at about body temperature in a short period from the viewpoint of the function, activity, and viability of the cells. Specifically, it is desirable to perform the thawing at 30°C to 40°C, preferably at 35°C to 38°C, and more

preferably at a temperature around human body temperature, for example, at about 37°C.

[0091] The cell aggregate frozen by the method of the present invention may be subjected to recovery culture after thawing by replacing the cryopreservation solution with a medium or may be transplanted into a living body without performing recovery culture.

[0092] That is, the cell aggregate frozen by the method of the present invention can maintain properties equivalent to those of an unfrozen cell aggregate. For example, the cell aggregate frozen by the method of the present invention and then subjected to recovery culture for 7 days after thawing has a marker expression rate equivalent to that of the cell aggregate before freezing. For example, in the case of the cell aggregate including dopamine-producing neuron progenitor cells, examples of markers expressed on the cells include FOXA2, LMX1A, NURR1 and TH. The equivalent marker expression rate means that the difference in numerical values of the percentages of the cells expressing a maker to the total cells between before freezing and after thawing, or after culturing for 7 days after thawing is about 10% or less.

[0093] The cell aggregate frozen by the method of the present invention is useful in that it can be transplanted into a living body without recovery culture.

[0094]

[Pharmaceutical composition]

The present application further provides a pharmaceutical composition, i.e., a composition (formulation) for transplantation, comprising, as an active ingredient, the cell aggregate frozen or preserved for a long-term by the above method.

[0095] The pharmaceutical composition (composition for transplantation) of the present invention is a concept including both a pharmaceutical composition frozen by the method of the present invention and a pharmaceutical composition obtained by thawing the frozen pharmaceutical composition. That is, examples of the pharmaceutical composition (composition for transplantation) of the present invention include a frozen or unfrozen composition comprising the cell aggregate including neural cells and the cryopreservation solution, as well as a composition comprising the cell aggregate including neural cells and a dosing vehicle where the cryopreservation solution has been replaced with the dosing vehicle after thawing.

[0096] Examples of the pharmaceutical composition (composition for transplantation) of the present invention include the compositions for transplantation according to [20] to [27] above.

[0097] Also, in one embodiment, the present application provides a frozen composition for transplantation, wherein the composition comprises:

a cell aggregate including 60% or more of dopamine-producing neuron progenitor cells and dopamine-producing

neurons derived from a pluripotent stem cell, having an equivalent spherical diameter of 150 μm to 1000 μm and including 500 to 150000 cells; and

a cryopreservation solution comprising 7% to 12% of dimethyl sulfoxide or propylene glycol and having the freezing point of -1°C to -10°C, the cryopreservation solution being preferably Bambanker hRM, and

wherein the frozen cell aggregate has following properties:

(1) about 60% or more of the total cells are viable after thawing;

(2) the cells after thawing have a neurite extension activity of 50% or more of that before freezing; and

(3) the rate of the FOXA2-positive cells, LMX1A-positive cells, NURR1-positive cells, and TH-positive cells in the cells viable after thawing are changed within ± 10% from those in the cells before freezing, preferably, the rate of the FOXA2-positive cells, LMX1A-positive cells, NURR1-positive cells, TH-positive cells, EN1-positive cells, and PITX3-positive cells are changed within ± 10%.

[0098] As one embodiment, the present invention encompasses a composition for transplantation wherein number of cells in the composition is 80000 to 5000000 cells/mL, 100000 to 4000000 cells/mL, or 200000 to 2000000 cells/mL, 300000 to 1000000 cells/mL, and the composition comprises FOXA2-positive and LMX1A-positive cells in an amount of 40% or more, preferably 60% or more, 60% or more,

80% or more, 85% or more, or 90% or more of the total cells, and TH-positive and NURR1-positive cells in an amount of 40% or less, 1% to 20%, or 5% to 15% of the total cells.

[0099] In one embodiment, the cell aggregate has an equivalent spherical diameter of 150 to 1000 μm, 150 μm to 600 μm, or 300 μm to 500 μm.

[0100] In one embodiment, the cell aggregate and the preservation solution have volume of 0.25 mL to 2 mL, 0.5 mL to 1.5 mL, or 0.5 mL to 1 mL.

[0101] In one embodiment, the cell aggregate and the preservation solution may be packed in a 0.5 mL to 15 mL, 0.5 mL to 5 mL, or 1 mL to 2 mL container.

[0102] As one embodiment, the present invention encompasses a composition for transplantation that can be used without recovery culture after thawing.

[0103] As one embodiment, the present invention encompasses the composition for transplantation according to any one of [20] to [24], wherein the composition comprises the cell aggregates in 8 to 192 cell aggregates/mL, the cell aggregates have an average particle size of 150 μm to 1000 μm, and number of cells per container is 80000 to 2400000.

[0104] The cell aggregate is useful as a pharmaceutical composition for transplantation for a patient suffering from a disease requiring transplantation of neural cells and can be used as a drug such as a therapeutic drug for

a disease accompanied by degeneration, injury, or dysfunction of neural cells. That is, a pharmaceutical composition comprising the cell aggregate of the present invention and a pharmaceutically acceptable carrier is also within the scope of the present invention.

[0105] Examples of the disease requiring transplantation of neural cells or the disease accompanied by injury or dysfunction of neural cells include spinal cord injury, motor nerve disease, multiple sclerosis, amyotrophic lateral sclerosis, atrophic lateral sclerosis, Huntington's disease, multiple system atrophy, spinocerebellar degeneration, Alzheimer's disease, Retinitis pigmentosa, age-related macular degeneration, and parkinsonian syndrome (including Parkinson's disease).

[0106] One embodiment of the present invention includes a pharmaceutical composition for treating Parkinson's disease, comprising, as an active ingredient, the cell aggregate of the present invention including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons. Number of dopamine-producing neuron progenitor cells and/or dopamine-producing neurons included in the therapeutic agent for Parkinson's disease is not particularly limited as long as the graft can be engrafted after the administration; for example, 1.0×10^4 cells or more can be included per transplantation. In addition, number of cells may be appropriately increased or decreased according to symptoms or body size to prepare

the therapeutic agent. Transplantation of the dopamine-producing neuron progenitor cells to a disease site can be performed, for example, by the technique described in Nature Neuroscience, 2, 1137 (1999) or N Engl J Med.; 344: 710 to 9 (2001).

[0107] As one embodiment, the pharmaceutical composition (also referred to as the composition for transplantation) of the present invention comprises a cell aggregate including neural cells to be transplanted into a human and a cryopreservation solution. The pharmaceutical composition of the present invention includes both a frozen solid form and a liquid form before freezing or after thawing. The pharmaceutical composition may include an additive used to maintain the survival of cells as appropriate, to such an extent that it will not affect the freezing rate and freezing temperature. Examples of the cryopreservation solution include those described above.

[0108] As described below, the pharmaceutical composition or the composition for transplantation of the present invention is used for transplantation after thawing it and removing and replacing the cryopreservation solution with a dosing vehicle injectable to a living body. That is, a composition comprising the thawed cell aggregate and the dosing vehicle is also within the scope of the pharmaceutical composition (also referred to as the composition for transplantation) of the present invention.

[0109]

[A method for producing a composition for transplantation]

The pharmaceutical composition (composition for transplantation) according to [20] to [27] above can be produced by the freezing method according to any one of [1] to [17] above. That is, the present invention encompasses a method for producing the above-described pharmaceutical composition (composition for transplantation).

[0110]

[Therapeutic method]

One embodiment of the present invention includes a method for treating a disease requiring supplementation of neural cells, which comprises the step of transplanting the cell aggregate of the present invention into a patient suffering from the disease requiring transplantation of neural cells.

[0111] In one embodiment of the present invention, the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons as obtained by the present invention can be administered to a patient with Parkinson's disease as a pharmaceutical composition, specifically as material for transplantation.

[0112] Specifically, the administration is performed as follows: the frozen pharmaceutical composition of the present invention comprising the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons, and the cryopreservation

solution is thawed, appropriately suspended in an appropriate medium for transplantation such as physiological saline, and then transplanted into a region of a patient where dopaminergic neurons are insufficient, for example, the corpus striatum. For example, the pharmaceutical composition after thawing may be washed with a vehicle comprising an appropriate carrier, and the cryopreservation solution may be replaced with a transplantation vehicle for suspending the cell aggregate for the transplantation into a human. A thawing temperature is not particularly limited but can be 30°C to 40°C, preferably 35°C to 38°C, and more preferably a temperature around human body temperature, for example, about 37°C as described above.

The cell aggregate included in the pharmaceutical composition (composition for transplantation) of the present invention can be transplanted into a living body by replacing the cryopreservation solution with a dosing vehicle without recovery culture after thawing.

[0113] For the carrier used for the transplantation vehicle (dosing vehicle) used for the cell aggregate including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons, any substance known to those skilled in the art can be used, as long as it is a substance used to maintain survival of cells. Specifically, a physiological aqueous solvent (such as physiological saline, a buffer solution, serum-free medium, etc.) can be

used. In transplantation medicine, if necessary, the pharmaceutical composition including tissue or cells to be transplanted may be combined with a preservative, a stabilizing agent, a reducing agent, or an isotonizing agent which is commonly used.

[0114] For the transplantation, the thawed cell aggregate may be preserved in a vehicle necessary to maintain the viability of each cell aggregate. Examples of the "vehicle necessary to maintain the viability" include medium and a physiological buffer solution; the vehicle is not particularly limited as long as the vehicle allows the cell population including dopamine-producing neuron progenitor cells and/or dopamine-producing neurons to survive, and those skilled in the art can appropriately select such a vehicle. One example of the vehicle is a medium prepared by using a medium usually used for culturing animal cells as a basal medium. Examples of the basal medium include media that can be used for culturing animal cells, such as BME medium, BGJb medium, CMRL 1066 medium, GMEM medium, Improved MEM Zinc Option medium, Neurobasal medium, IMDM medium, Medium 199 medium, Eagle MEM medium, αMEM medium, DMEM medium, F-12 medium, DMEM/F12 medium, IMDM/F12 medium, Ham medium, RPMI 1640 medium, Fischer's medium or mixed media thereof.

[0115] Due to the transplantation of the above cell aggregate, the transplanted dopamine-producing neuron progenitor cells and/or dopamine-producing neurons as well

as dopamine-producing neuron progenitor cells and/or dopamine-producing neurons that are induced after the transplantation are functionally engrafted in the patient to which the cell aggregate is administered.

[0116] The term "engraftment" as used herein means that transplanted cells survive in a living body for a long period of time (e.g., 30 days or more, 60 days or more, or 90 days or more) and adhere and remain in an organ.

[0117] The term "functional engraftment" as used herein means a state in which transplanted cells are engrafted to perform their original function in a living body.

[0118] The term "functional survival rate" as used herein refers to the percentage of the cells that have achieved functional engraftment in the transplanted cells. The functional survival rate of transplanted dopamine-producing neuron progenitor cells can be determined, for example, by measuring number of TH-positive cells in the graft.

[0119] Due to the transplantation of the above cell aggregate, the transplanted cells and the dopamine-producing neuron progenitor cells and/or dopamine-producing neurons induced after the transplantation have a functional survival rate of 0.1% or more, preferably 0.2% or more, more preferably 0.4% or more, even more preferably 0.5% or more, and still more preferably 0.6% or more.

[0120] As one embodiment, the present invention encompasses a method for treating a disease requiring regeneration of a dopamine-producing nerve, which comprises following steps of:

(1) thawing the composition for transplantation according to any one of [20] to [27] at 30°C to 40°C, preferably at 37°C ± 3°C; and

(2) transplanting the composition for transplantation obtained in (1) into a corpus striatum region of a patient.

One embodiment of the present invention encompasses the treating method, wherein the cryopreservation solution is replaced with a dosing vehicle without culture after thawing to perform step (2).

[0121] Examples of a mammal to undergo the transplantation in the present specification include humans, mice, rats, guinea pigs, hamsters, rabbits, cats, dogs, sheep, pigs, cows, horses, goats, and monkeys, preferably rodents (e.g., mice and rats) or primates (e.g., humans and monkeys), and more preferably humans.

EXAMPLES

[0122] Hereinafter, the present application is described in more detail with reference to Examples but is not limited to the Examples.

Example 1

An outline of Example 1 is shown in Figure 1.

[0123]

[Material and method]

Maintenance and neural differentiation of human iPS cells

In accordance with the dual SMAD inhibition and floor plate induction protocol described in Doi et al., 2014, iPSC-derived dopamine-producing neuron progenitor cells were inducted. That is, human iPSCs (1231A3) (Kyoto University) were maintained in Stem Fit medium (AJINOMOTO CO., INC.) on a 6-well plate coated with iMatrix-511 (Nippi, Inc.). To initiate neuronal differentiation, the iPSCs were incubated with TrypLE select (Invitrogen) for 10 minutes, then dissociated into single cells and seeded with differentiation medium at a density of 5×10^6 cells/well on the 6-well plate coated with iMatrix-511 (Nippi, Inc.). The differentiation medium was medium containing GMEM supplemented with 8% KSR, 0.1 mM MEM non-essential amino acids (all from Invitrogen), sodium pyruvate (Sigma-Aldrich), and 0.1 mM 2-mercaptoethanol. The differentiation medium was changed every day from the day after seeding until day 12. To increase cell viability after seeding, 10 μM Y-27632 (WAKO CHEMICAL CO., LTD.) was added on day 1. To efficiently induce the neuronal differentiation, LDN193189 (STEMGENT, INC.) and A83-01 (WAKO CHEMICAL CO., LTD.) were added. To induce floor plate cells, 2 μM Purmorphamine and 100 ng mL^{-1} FGF8 (WAKO CHEMICAL CO., LTD.) were added on days 1 to 7, and 3 μM CHIR99021 (WAKO CHEMICAL CO., LTD.) was added on days 3 to 12 (see Figure 1).

[0124]

Cell sorting and culture

On day 12, cells expressing CORIN (a floor plate marker in the developing brain) were isolated to concentrate the dopamine-producing neuron progenitor cells, and sorting (FACS technique) was performed thereon. First, cultured cells were stained with PE-labeled anti-CORIN antibody (100ng/mL; Catalent/BD) for 20 minutes. Dead cells and debris were excluded through 7-AAD staining. Analysis was performed using a FACSAria II or III cell sorter and FACSDiva software program (BD Biosciences). After cell sorting on day 12 of culture, the sorted cells were reseeded at a density of 2-3 × 10^4 cells/well in neuronal differentiation medium containing neurobasal medium supplemented with B27 supplement, 2 μM Glutamax-I (all from Invitrogen), 10 ng mL^{-1} GDNF, 200 mM ascorbic acid, 20 ng mL^{-1} BDNF (all from WAKO CHEMICAL CO., LTD.) and 400 μM dbcAMP (Sigma-Aldrich) on a low cell adhesion U-shaped 96-well plate (Sumitomo Bakelite Co., Ltd.), and cultured as floating spheres until day 28. Unsorted cells were also reseeded at a density of 1.5 × 10^4 cells/well. Half of the medium was replaced every 3 days, and 30 μM Y-27632 (WAKO CHEMICAL CO., LTD.) was added only to the first medium. For prolonged culture, the floating spheres were cultured in the neuronal differentiation medium (see Figure 1).

[0125]

Cryopreservation method

The spheres collected on day 28 were cryopreserved and used in subsequent experiments. That is, the collected spheres were placed in cryovials containing 1 mL of ice-cold cryopreservation solution shown in Table 1 and kept on ice until freezing. For cryopreservation, the vials were transferred into a freezing container: BICELL (NIHON FREEZER CO., LTD.), a programmed freezer: PDF-150, 250 (STREX Inc.), cryomed (Thermo Fisher Scientific Inc.) or a proton freezer (RYOHO FREEZE SYSTEMS. CO., LTD.). Six different cooling profiles (shown in Figure 3) were used in this experiment. That is, BICELL was transferred into a deep freezer (-80°C) and kept for 4 hours or more (upper left in Figure 3). In the controlled-rate freezing method, the vials were frozen to -40°C at a rate of 0.5 °C/min (upper center in Figure 3) or 1 °C/min (upper right in Figure 3) and then cooled to -80°C at a faster rate of 3 to 5 °C/min. In the shock cooling method, the procedure of freezing to -35°C at a rate of -25°C/min and subsequent warming to -12°C at a rate of +10°C/min was inserted at a temperature of -4°C during freezing (lower left and center in Figure 3). The vials frozen with the proton freezer were kept in a chamber for 30 to 60 minutes (lower right in Figure 3). The proton freezer has a static magnetic field, electromagnetic waves, and cold air in combination. It is considered that static magnetic fields and electromagnetic fields influence the orientation of water

molecules and cause the formation of small ice crystals, thereby preventing cell disruption. However, these mechanisms have not yet been fully understood. After freezing, the cryovials were preserved in the vapor phase of a liquid nitrogen tank. The frozen cells were thawed at 37°C for about 2 minutes and transferred into a 15 mL tube containing 10 mL of neurobasal medium. After removal of the supernatant, the cells were rinsed with PBS and used for each assay or transplantation. To estimate number of cells after cryopreservation, about 50 aggregates were dissociated and counted with a hemacytometer to calculate the cell concentration one day after thawing and before freezing.

Table 1 Commercially available xeno-free cryopreservation solutions used in the present Examples

[Table 1]

Medium	Brand name	Supplier	Mejor component
SCB	STEM-CELLBANKER	ZENOAQ	10 % DMSO
SCB DMSO free	STEM-CELLBANKER DMSO free	ZENOAQ	10 % Propylene glycol
BBK	Bambanker hRM	NIPPON Genetics	10 % DMSO
CS5	Cryostor CS5	BioLife Solutions	5 % DMSO
CS10	Cryostor CS10	BioLife Solutions	10 % DMSO
SaF	Synth-a-Freeze	Thermo Fisher Scientific	10 % DMSO

[0126]

Quantitative RT-PCR

Total RNA was extracted from undifferentiated cells, iPSC-derived dopamine-producing neurons (on day 28, day 29, day 31, and day 35), and iPSC-derived dopamine-

producing neurons after thawing on days 28 + 0, 1, 3, and 7 using RNeasy Mini Kit or RNeasy Micro Kit (Qiagen) and cDNA was synthesized using Super Script III First-Strand Synthesis System (Invitrogen). Quantitative PCR reactions were performed by using Power SYBR Green PCR Master Mix (Applied Biosystems) with StepOne. Data were evaluated using the delta Ct method and normalized by GAPDH expression. The primer sequences used are shown in Table 2.

[Table 2]

Gene	Forward	Reverse
POU5F1	AGACCATCTGCCGCTTTGAG (SEQ ID NO: 1)	GCAAGGGCCGCAGCTT (SEQ ID NO: 2)
NANOG	GGCTCTGTTTTGCTATATCCCCTAA (SEQ ID NO: 3)	CATTACGATGCAGCAAATACGAGA (SEQ ID NO: 4)
FOXA2	TTCAGGCCCGGCTAACTCT (SEQ ID NO: 5)	AGTCTCGACCCCCACTTGCT (SEQ ID NO: 6)
LMX1A	GATCCCTTCCGACAGGGTCTC (SEQ ID NO: 7)	GGTTTCCCACTCTGGACTGC (SEQ ID NO: 8)
EN1	TGGGTGTACTGCACACGTTATTC (SEQ ID NO: 9)	GGAACTCCGCCTTGAGTCTCT (SEQ ID NO: 10)
NURR1	CGAAACCGAAGAGCCCACAGGA (SEQ ID NO: 11)	GGTCATAGCCGGGTTGGAGTCG (SEQ ID NO: 12)
PITX3	GGGCCAGGAGCACAGCGACTCA (SEQ ID NO: 13)	GCTGCCGCCGCTGCTTCTTTTT (SEQ ID NO: 14)
TH	GCAGTTCTCGCAGGACATTG (SEQ ID NO: 15)	CGGCACCATAGGCCTTCA (SEQ ID NO: 16)
TPH2	TCAGCTACTTGGCAGCTCAAC (SEQ ID NO: 17)	CTTGCCACTTTCGGTAGCAG (SEQ ID NO: 18)
GAPDH	GGTCGGAGTCAACGGATTTG (SEQ ID NO: 19)	TCAGCCTTGACGGTGCCATG (SEQ ID NO: 20)

[0127]

Immunofluorescence studies

In *in vitro* studies, cultured cells on day 35 or on day 7 after thawing were fixed with 4% paraformaldehyde.

In the *in vivo* studies, fixed and frozen brains were sliced into 40-μm thin slices. The slices were immunostained using the free-floating method. The primary antibodies used are shown in Table 3. Cells were visualized using a fluorescence microscope (BZ-9000; Keyence Corporation) and a confocal laser scanning microscope (Fluoview FV1000D; Olympus). Number of immunoreactive cells was quantified in every 6th sectionthroughout the grafts and corrected using the Abercrombie method.

[Table 3]

Antibody	Species	Dilution	Supplier	Catalog#
TH	Rabbit	1:400	Millipore	AB152
NURR1	Rat	1:1000	Donated by KAN laboratory	-
FOXA2	Goat	1:500	R&D systems	AH2400
SOX1	Goat	1:100	R&D systems	AH3369
PAX6	Mouse	1:500	BD Pharmingen	561462
KI67	Rabbit	1:1000	Novocastra	NCLKi67P
TUBB3	Mouse	1:400	Covance	MMS-435P
HNA	Mouse	1:500	Millipore	MAB1281

*TUBB3 indicates βIII-Tubulin.

[0128]

Neurite extension assay

On day 28, floating spheres picked for a neurite extension assay were cultured on a 24-well plate coated

with iMatrix-511 for 5 days and fixed with 4% paraformaldehyde. The spheres were stained with PE-labeled anti-PSA-NCAM antibody (1:100; Miltenyi Biotec) and visualized using a fluorescence microscope (BZ-9000; Keyence). The area of PSA-NCAM-positive neurites without cell bodies was measured using Photoshop (Adobe systems) and WinRoof (Mitani Corporation).

[0129]

Electrophysiologic analysis

The suspension spheres were dissociated with papain on day 28 and cultured on a plate coated with poly-l-ornithine, fibronectin, and laminin (O/F/L) until day 49. Nerves with large cell bodies and neurite-like structures were selected for whole-cell patch-clamp recording. The cells were maintained in a physiological saline solution containing following components: 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, and 17 mM glucose. A patch pipette was prepared from a borosilicate glass capillary (GC150TF-10; Clark). This patch pipette had a resistance of 3 to 4 MW when filled with an internal solution composed of 140 mM KCl, 10 mM HEPES, and 0.2 mM EGTA (pH 7.3). Voltage-clamp and current-clamp recordings were performed using a patch-clamp amplifier (EPC-8; HEKA). The gigaseal resistances were in the range of 10 to 20 GW. The current signals from a patch clamp amplifier were filtered at 5 kHz through a 4-pole low-pass filter (UF-BL2; NF) having the Bessel

characteristics, sampled using a 12-bit A/D converter, and stored on a 32-bit computer (PC-9821Ra333; NEC). All experiments were performed at room temperature.

[0130]

Dopamine release assay

On day 28, floating spheres picked for a dopamine release assay were cultured on a 12-well plate coated with O/F/L for another 28 days, washed twice with a solution having a low concentration of KCl (4.7 mM), and incubated in a solution having a low concentration of KCl for 15 minutes. The medium was then replaced with a solution having a high concentration of KCl (60 mM) for 15 minutes. The solution was collected, and its dopamine concentration was determined by LC/MS/MS. The cells remaining on the plate were collected in PBS and sonicated. The DNA concentration of a cell lysate was measured using the Quant-iT ™ dsDNA Assay Kit (Thermo Fisher Scientific Inc.) and used to correct the dopamine concentration.

[0131]

Microarray analysis

A cDNA microarray analysis was performed in the Bio-Medical Department of Kurabo Industries Ltd. The total RNA of the undifferentiated cells, the cultured cell on day 12, the iPSC-derived dopamine-producing neurons (day 28, day 29, day 31, and day 35), and the iPSC-derived dopamine-producing neurons after thawing on days 28 + 0, 1, 3, and 7 was processed using Genechip® 3'IVT pico

Reagent Kit and Human Genome U133 Plus 2.0 array (Affymetrix, Inc.). Data analysis was performed using Genechip Operating Software ver. 1.4 (Affymetrix, Inc.). Signal detection and quantification was performed using MAS5 algorithm. Global normalization was performed such that the average signal intensity of all probe sets was equal to 100. Analysis was performed using data on a probe set showing a signal intensity higher than 50 at $p < 0.05$ and showing a 2-fold or more change between the samples. [0132]

Cell transplantation

Experimental animals were treated and handled in accordance with the Guidelines for Animal Experiments of Sumitomo Dainippon Pharma Co., Ltd., Guidelines for Animal Experiments of Kyoto University, and the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources (ILAR; Washington, DCA). Male F344/NJcl-rnu/rnu (nude) rats, Parkinson's disease model F344 NJcl-rnu/rnu rats (CLEA Japan, Inc.), and Sprague Dawley (SD) rats (SHIMIZU LABORATORY SUPPLIES Co., Ltd.) were used for a short-term transplantation study. The SD rats were injected with 6-OHDA into the medial forebrain bundle in the right hemisphere at following coordinates (A, -4.0; L, -1.3; V, -7.0). A total of 19.2 mg of 6-OHDA (in 3 μL of physiological saline containing 0.02% ascorbic acid) was injected into each rat. From one day prior to transplantation, the SD rats were immunosuppressed daily

with cyclosporine (10 mg/kg, i.p., LC Laboratories). Cell transplantation was performed with stereotactic injection of the spheres (A, +1.0; L, -3.0; V, -5.0 and -4.0; as well as TB, 0 (2 μl; 200,000 cells/μl).

[0133] Nude rat models for Parkinson's disease were used for a long-term transplantation study. Cell transplantation was performed with stereotactic injection of the unfrozen spheres (A, +1.0; L, -3.0; V, -5.0 and -4.0; as well as TB, 0 (2μl; 200,000 cells/μl) or the cryopreserved spheres (A, +1.0; L, -3.5 and 2.5; V, -5.5 and -4.5; as well as TB, 0 (4μl; 200,000 cells/μl) into the corpus striatum of the right brain. The experimental animals were transcardially anesthetized and perfused with PBS followed by 4% paraformaldehyde.

[0134]

Behavioral analysis

An evaluation of a methamphetamine-induced rotational movement was performed before the transplantation and every 4 weeks after the transplantation using rotation bowls monitored with a video. Methamphetamine (Sumitomo Dainippon Pharma Co., Ltd.) at a dose of 2.5 mg/kg was injected intraperitoneally and rotations were recorded for 90 minutes.

[0135]

Statistical analysis

Statistical analysis was performed using a commercially-available software package (GraphPad Prism 6;

GraphPad Software). Data from *in vitro* were analyzed by the one-way ANOVA and Tukey's post hoc analysis (Figures 2A, 2B, 4A, 4B, 5A, and 5B). Behavioral data were analyzed by two-way ANOVA with Tukey's multiple comparison test (Figure 8A). The differences were considered to be statistically significant for values of $p < 0.05$. The data were presented as mean ± SD, except for the behavioral data (mean ± SEM).

[0136]

[Result]

(1) Examination result on cryopreservation solutions (Reference Example)

To establish the cryopreservation conditions suitable for iPSC-derived dopamine-producing neuron progenitor spheres as described above, clinically applicable cryopreservation solutions listed in Table 1 above were compared.

[0137] First, as described in Doi et al., 2014, conditions for a cryopreservation solution were screened using neural progenitor spheres that were not sorted by CORIN. It has been reported that number of cells reduces after thawing due to necrosis and subsequent persistence of delayed apoptosis and delayed necrosis (Milosevic et al., 2005). It is known that the proliferative capacity begins to recover during this process and number of cells bottoms out about 24 to 48 hours after thawing (Malpique et al., 2010; Mitchell et al., 2014; Baust et al., 2017). It has

also been reported that neurotoxic compounds or diseases related to genetic aberration can cause neurite abnormality (Radio et al., 2008; Reinhardt et al., 2013; Ryan et al., 2016). These phenomena suggest that neurite morphology is a good indicator of characteristics of a neuron. Therefore, the recovery rate of viable cells one day after thawing was evaluated as cell viability, and also the area of neurite extension was examined as a new functional evaluation. The results are shown in Figure 2A, Figure 2B, and Figure 2C.

[0138] In all cryopreservation solutions, the cell viability and neurite extension significantly reduced after freezing (Figure 2). All the preservation solutions with 10% DMSO showed significantly higher viability than STEM-CELL BANKER DMSO free (SCB DMSO free) (21 ± 7%) and CryoStor CS5 (CS5) (16 ± 6%) (Figure 2A). Bambanker hRM (BBK) and Synth-a-Freeze (SaF) showed a tendency of relatively greater neurite extension than the other freezing conditions (Figure 2B and Figure 2C). 8 ± 17%, 50 ± 43%, and 13 ± 25% of the spheres did not adhere to the plate in the SCB DMSO free condition, CS5 condition, and CryoStor CS10 condition, respectively. Since BBK was already registered in the Drug Master File in Japan, BBK was used in following experiments.

[0139]

(2) Effects of intermediate speed cooling and suppression of supercooling

The unsorted spheres in BBK were then cryopreserved using six different protocols. The temperature profile for each protocol is shown in Figure 3. In addition, a shock cooling method having a process of transient temperature drop (Morris and Acton., 2013) was also examined in order to verify the effect of controlling ice nucleation and latent heat at the freezing point. Proton freezers have the distinctive cooling profile in addition to magnetic field and electromagnetic waves. Proton freezers cool a sample from -4°C to -30°C at about 5°C/min without supercooling. This cooling rate is intermediate between the conventional slow cooling method and the vitrification method. Specifically, in the case of cooling in a proton freezer, the sample in the chamber is cooled at about 2 to 7°C/min as an actual value.

[0140] The conditions of the proton freezer and 0.5°C/min and 1°C/min without shock cooling process resulted in relatively higher cell viability than the other conditions (Figure 4A). In particular, the conditions of the proton freezer and 0.5°C/min showed about 80% cell viability compared to unfrozen cells. Also, the proton freezer showed significantly greater neurite extension than the other freezing conditions, except for the condition of 1°C/min with the shock cooling process, which was about 60% extension compared to that of the unfrozen cells (Figure 4B). These results demonstrate the maintenance of cellular functions in the condition of the proton freezer.

When the time for immersion in the cryoprotectant before freezing was extended to 1 hour, good results for cell viability and neurite extension was obtained under the proton freezer condition as in the case of the immersion time of 15 minutes (Figures 5A and B). In addition, the cell viability was reduced under the condition of 0.5°C/min, but no significant differences were observed for cell viability or neurite extension under the other conditions (Figures 5A and 5B). Therefore, it was found that the intermediate speed cooling and suppression of supercooling are effective for cryopreservation of iPSC-derived dopamine-producing neuron progenitor spheres.

The spheres used above were induced in the same manner as in Figure 1, except that sorting with anti-CORIN antibody was not performed. The results of measuring the expression of the markers in the spheres before freezing are shown in Figure 10 and Table 4.

[Table 4]

	Percentage relative to DAPI-positive cells
FOXA2/LMX1A	84.9±5.5%
NURR1	21.2±11.3%
TH	7.4±2.7%
SOX1	4.8±2.0%
PAX6	2.6±1.0%
KI67	8.5±1.8%

[0141]

(3) Cell number and function of cryopreserved iPSC-derived dopamine-producing neuron progenitor spheres

Based on the above screening results, the properties of the iPSC-derived dopamine-producing neuron progenitor spheres cryopreserved using BBK and the proton freezer were examined after thawing. In order to concentrate the iPSC-derived dopamine-producing neuron progenitor cells to 80% or more, the cells were sorted using CORIN antibody, and subjected to suspension culture under the above-described conditions to form cell aggregates, followed by cryopreservation using BBK and a proton freezer (see Figure 1). The immersion time in BBK was 1 hour.

[0142] As a result, the cryopreserved spheres were 200 μm to 500 μm in size, and number of cells was 5500 to 12000 (the number of cells was not measured in all spheres and is estimated to be 2000 to 15000 in view of size of the spheres). The resulting spheres showed a cell viability of 52 ± 8% and a neurite extension of 51 ± 19% (Figures 6A, B). To confirm that the spheres are dopamine-producing neuron progenitor cells, protein markers and gene expression were examined 7 days after thawing. As a result of immunocytochemistry, it was found that the cryopreserved spheres expressed almost the same level of the dopamine-producing neuron progenitor cell marker (FOXA2), the dopamine-producing nerve markers (NURR1 and TH), and the proliferating cell marker (KI67) as compared with the unfrozen spheres (Figures 6C to E).

Because the dopamine-producing neuron progenitor cells were highly concentrated by the cell sorting process,

cells expressing neural stem cell markers (SOX1 and PAX6) were quite few in both unfrozen and frozen spheres. This indicates that no abnormal proliferation were occurred by the cryopreservation process. Further, a qPCR analysis showed that the expression levels of dopamine-producing neuron progenitor cell markers (FOXA2, LMX1A, and EN1) and dopamine-producing nerve markers (NURR1, PITX3, and TH) were not changed between with or without cryopreservation (Figure 6G). The expression level of pluripotency markers (POU5F1 and NANOG) was maintained less than 1% of day 0 in both spheres (Figure 6F).

[0143] In addition, Figure 11 shows the marker expression of the sphere immediately before freezing in the same lot as the sphere shown in Figure 6C, and Table 5 shows the results of measuring the marker expression in multiple lots.

[Table 5]

Lot	1	2	3	4	Average	SD
SOX1	0.0	0.1	1.1	0.0	0.3	0.5
PAX6	0.0	0.0	0.0	0.5	0.1	0.3
KI67	20.0	20.1	7.7	16.9	16.2	5.8
NURR1	27.8	38.9	8.5	40.8	29.0	14.8
TH	13.3	12.4	9.6	12.0	11.8	1.6

Also, the expression rates of FOXA2 and LMX1A were compared (Table 6).

[Table 6]

LOT	1	2	3	4	Average	SD
LMX1A	86.5	87.8	64.8	92.6	82.9	12.4
FOXA2	80.9	88.1	70.3	90.8	82.5	9.1
LMX1A/FOXA2	78.5	82.6	57.7	85.5	76.1	12.6

[0144] A global PCA analysis using the microarray data was also performed to overview the temporal changes during maturation after the cryopreservation/thawing process. A total of 21,882 probe sets containing significant signals were used for the analysis. The PC1 and PC2 contributions were 48.0% and 15.7%, respectively. PC score plots were clustered into three main groups (day 0 (iPSC), day 12, and after day 28) (Figure 6H). The PCA plots for both unfrozen samples and cryopreserved samples shifted in the positive direction on the PC1 and PC2 axes, regardless of cryopreservation on day 28 and after day 28. Only 206 (0.88%) out of 23349 probe sets showed an increase or decrease more than 2-fold between the unfrozen samples and the cryopreserved samples in the same differentiation batch. In contrast, 222 (0.95%) out of 23275 probe sets showed an increase or decrease more than 2-fold between the unfrozen samples in different batches (Figures 6I, J). This result strongly indicates that the cryopreservation process has not affected the cell properties of the dopamine-producing neuron progenitor cells.

[0145] To confirm functional maturation of the iPSC-derived dopamine-producing neuron progenitor spheres, an

electrophysiological analysis and a dopamine release measurement were performed. When the cryopreserved spheres were dissociated and cultured on the plate for further maturation, most of the cells gave rise to $TH^+/TUBB3^+$ dopamine-producing nerves on day 28 + 21 (Figure 6K). At this time point, continuous action potentials from the mature dopamine-producing nerves were detected by the whole-cell patch clamp technique, in which current is kept constant (Figure 6L). Dopamine secretion in the unfrozen spheres on day 56 and in the cryopreserved spheres on day 28 + 28 was also detected by LC/MS/MS. The amount of released dopamine was comparable to that of the unfrozen sphere (Figure 6M). Therefore, it was found that cell number and function of the cryopreserved iPSC-derived dopamine-producing neuron progenitor spheres are maintained.

[0146]

(4) Engraftment of cryopreserved iPSC-derived dopamine-producing neuron progenitor spheres, and behavior of 6-OHDA lesioned rats

To confirm that the cryopreserved iPSC-derived dopamine-producing neuron progenitor cells could survive after transplantation, the unfrozen sphere and the cryopreserved sphere were transplanted into several types of rat corpus striatum and observed for a short period, followed by determination of a graft survival rate and number of survived cells. The results are shown in Table

7. There was no difference in number of rats with surviving grafts between both of the groups. When the spheres cryopreserved with a proton freezer and BBK were transplanted, the percentage of total surviving cells (HNA^+) was 42 ± 20% of that of the unfrozen cells.

Table 7. Results of a short-term validation experiment evaluating the graft survival rate of the cryopreserved spheres.

[Table 7]

	Animal		Duration	Number of animals with cells		Average number of survived cells	
	strain	Model		Unfrozen	Frozen	Unfrozen	Frozen
1383D6	Nude	Nomal	1 month	3/3	3/3	25555	4554
1231A3	Nude	Nomal	1 month	2/3	2/3	13589	8330
FF-I01	SD	6-OHDA lesioned	3 months	2/2	1/1	31781	18326
FF-I01	Nude	6-OHDA lesioned	2 weeks	3/3	3/3	31685	11520

[0147] The results of a short-term validation experiment with transplantation indicate that more than twice cryopreserved cells should be transplanted as compared to the unfrozen cells in order to achieve comparable number of survived cells *in vivo*. Therefore, to evaluate number of survived cryopreserved cells and their pharmacological effect, the unfrozen spheres (4×10^5 cells) and the cryopreserved spheres (8×10^5 cells) were transplanted into 6-OHDA lesioned PD disease model rats to measure their methamphetamine-induced rotational movements. Twenty four weeks after the transplantation, abnormal rotations were reduced in both of the groups (Figure 8A). Immunofluorescent staining at 24 weeks confirmed cell

engraftment in all rats that showed behavioral recovery. 55996 ± 3603 HNA^+ cells were survived in the graft derived from the unfrozen spheres, and 36486 ± 3578 HNA^+ cells were survived in the graft derived from the cryopreserved spheres (Figures 8B, C). For number of survived HNA^+ cells, no significant difference was found between both of the groups. Both of the groups had TH^+ cells survived and showed normal neurite extension (Figures 8D to G). There was no difference in number of TH^+ cells between the graft derived from the unfrozen spheres and the graft derived from the cryopreserved spheres (3603 ± 1576 cells/graft and 3578 ± 1490 cells/graft) (Figure 8H). TH^+ cells survived in the grafts derived from the unfrozen spheres and the cryopreserved spheres were 1.1 ± 0.4% and 0.5 ± 0.2% of injected cells, respectively. This result means that about 50% of TH^+ nerves survived in the graft derived from the cryopreserved spheres compared to that of the unfrozen spheres. Further, the most of the surviving cells in both of the groups were $FOXA2^+$ dopamine-producing neuron progenitor cells: 71 ± 6% for the unfrozen spheres and 76 ± 4% for the cryopreserved spheres (Figures 8I, J). In contrast, $KI67^+$ proliferating cells, which may cause the risk of abnormal proliferation, were quite few in both of the groups (Figures 8I, K). Therefore, it was found that the cryopreserved iPSC-derived dopamine-producing neuron progenitor spheres are engrafted and improve the behavior of 6-OHDA lesioned rats.

[0148]

(5) Expression of dopamine-producing neuron progenitor cell markers

To confirm that the thawed cells have the ability to mature into dopamine-producing neurons similar to the unfrozen cells, protein markers and gene expression were examined over time until 7 days after thawing. As a result of immunocytochemistry, it was found that the cryopreserved spheres continued to express almost the same level of FOXA2 as compared to the unfrozen spheres (Figure 7A). NURR1+ cells increase from day 28 to day 35 in unfrozen cells. The cells after thawing showed the increase of NURR1+ cells at 7 days after thawing, which was slightly later than the unfrozen cells (Figure 7B). Further, a qPCR analysis showed that the expression level of TH was increased in the unfrozen cells with culturing period until day 35. It was confirmed that the expression of TH remained constant in the cells from day 0 to day 3 after thawing, and was increased on day 7 (Figure 7C). Therefore, it was found that the cryopreserved iPSC-derived dopamine-producing neuron progenitor spheres maintain the ability to mature into dopamine-producing neurons.

In addition, Table 8 shows the results of comparing unfrozen cells cultured up to day 35 with the cells frozen on day 28 and cultured for 7 days after thawing in multiple lots.

[Table 8]

LOT		1	2	3	4	Average	SD
D35	SOX1	0.5	0.0	0.0	0.3	0.2	0.2
	PAX6	0.0	0.0	0.4	0.2	0.1	0.2
	KI67	10.4	6.1	4.3	8.4	7.3	2.7
	FOXA2	82.9	90.4	83.0	88.8	86.3	3.9
	NURR1	36.8	51.3	9.5	28.5	31.5	17.5
	TH	27.0	18.4	18.8	15.8	20.0	4.9
D28+7	SOX1	0.0	0.4	0.0	0.0	0.1	0.2
	PAX6	0.0	0.0	0.5	0.0	0.1	0.3
	KI67	13.3	7.5	16.9	4.6	10.6	5.5
	FOXA2	83.2	88.8	81.1	82.0	83.8	3.5
	NURR1	44.7	41.0	23.9	46.1	38.9	10.2
	TH	15.9	9.0	23.3	20.2	17.1	6.2

[0149]

(6) Comparison of thawing conditions (reference)

The thawing conditions for cryopreserved spheres frozen with a proton freezer using BBK as a cryopreservation solution were examined. The tubes stored in the vapor phase of liquid nitrogen were taken out and thawed under three conditions of: (i) room temperature; (ii) a 37°C water bath; and (iii) melting at about 3°C/min (actual value) using a programmed freezer. The times required for thawing were (i) 17 minutes, (ii) 2 minutes, and (iii) about 25 minutes, respectively. The survival rate (recovery of viable cells) and neurite extension activity of the spheres after thawing were measured by the methods described above. As shown in Figure 9, thawing in the 37°C water bath gave the best result.

Appendix B – PROTON Patents (original documents)

1. **EP 4063496 A1:** *"Method for cryopreserving biological material using electromagnetic fields"* . European publication available in EPO records. Includes a complete description of the PROTON device, schematic figures of the configuration (coils, uniform magnetic field, cold air flow) and examples of implementation in neural neurospheres.
   SEE ANNEX A-4

2. **JP 2025094837 A:** *"磁場および電場を用いた細胞凍結方法"* (in Japanese, with English translation available). J-PlatPat document detailing the method steps in the context of neural iPSCs. Includes laboratory data such as viability vs. field strength tables.
   SEE ANNEX A-3

3. **US 2023000071 A1:** *"Electromagnetic freezing method for cells and tissues"* . Published in the USPTO, generally in English, with claims possibly analogous to those of JP. Consult for those requiring the legal English wording of the PROTON method.
   SEE ANNEX A-5

Annex C – Technical specifications of the PROTON P-15 NEO equipment

PROTON equipment is manufactured by **Ryoho Freeze Systems Co. (Japan)** and distributed in Europe by PROTON Europe SL. Below are some indicative specifications (biomedical model **PF-15 NEO** , *" High-grade model") . for medical field "*):

Figure 1: ***PROTON Freezer PF-15 NEO equipment*** *(biomedical model with a nominal capacity of 15 kg/h)*

Technical Data Sheet – PROTON *PF-15 NEO Magnetic Freezer*

This freezer from Ryoho Freeze Systems (Japan) combines a sanitary stainless steel design with electromagnetic coils integrated into the cooling chamber.
The illustrated model, of small industrial size, is designed to facilitate its integration into GMP environments and allows large batches of cell samples to be frozen uniformly.
It is based on the model PF-15C for food freezing but the PF-15 NEO version incorporates a specific electromagnetic system (static magnetic field + RF) not present in the standard model.

The **technical characteristics of the PF-15 NEO** are:

- **Freezing capacity:**

~15 kg/hour of biological material per cycle (enough to process around *150 1 mL vials* per run, depending on the heat load).
This high capacity ensures scalability for cell banks or clinical batch production.

- **Operating temperature range:**

Typical operating range: -30 °C to -35 °C in standard mode.
Cooling is achieved through a cold **forced air system** with precise *PID control* , ensuring stable and homogeneous temperature ramps .
Precise thermal control using a digital PID system with homogeneous stability in the chamber.

- **Electromagnetic fields:**

It includes:
  - A **uniform static magnetic field** generated by permanent magnets (or equivalent electromagnet).

This field aligns the water molecules during cooling.
  - A **radio frequency** (RF) electric field generated by emitting plates

The RF field is applied simultaneously to the magnetic field to induce multiple nucleation.
Both fields are adjustable to low/medium/high levels according to predefined protocols, or exact parameters for research.

- **Control and programming:**

It has an integrated digital panel and/or external software to program custom **cooling profiles** (with ramps, plateaus, rates in °C/min).
It also allows you to control the intensity of the EM fields during each segment of the program. It has internal memory to store multiple validated programs and automatically records temperature-time data for each cycle (with the option to export reports for GMP compliance).
An electromagnetic field parameter recorder is available as an option.

- **Physical dimensions:**

Approximately **1.5 m (width) × 0.8 m (depth) × 1.8 m (height)** in the PF-15 NEO 15 kg/h model. (In Japanese specifications equivalent: 1200 mm × 870 mm × 1700 mm.)
Weight ~450 kg including the magnetic and cooling system.
The exterior is pharmaceutical-grade stainless steel, with smooth surfaces and rounded corners for easy cleaning.

- **Internal camera and trays:**

Usable dimensions of the chamber ~ **600 mm × 400 mm × 350 mm** , with capacity for **5** stacked special metal trays (of ~600 × 400 mm each).
These trays allow vials, bags, or other containers to be layered, ensuring uniform airflow around each sample (Maximum height per unit: 350 mm)
The chamber has a double-sealed door with high-performance insulation to maintain the temperature and prevent moisture from entering.

- **Cooling systems:**

Use an industrial-grade refrigeration compressor with environmentally friendly refrigerant (e.g., R-448A). Cooling is fast and controlled; typical time to go from ambient temperature to -40 °C is <30 min without load.
The system is designed to prevent excessive vibrations or sudden temperature changes.

- **Electrical requirements and consumption:**
  - AC200V power supply , **three-phase** , 50/60 Hz
  - Requires **nominal current:** 16.2A (50Hz) / 16.15A (60Hz)
  - **Recommended breaker:** 20th
  - **Electrical consumption:** 3.4 kW (50Hz) / 3.9 kW (60Hz)

Note: The equipment does not require water connections or drains; only adequate room ventilation to dissipate ~5 kW of waste heat.

- **Laboratory/GMP integration:**

The equipment has **CE certification** for electrical safety and electromagnetic compatibility.
It is designed for use in controlled environments: all materials in contact with samples (e.g. racks, trays) are **sterilizable** (autoclavable or disinfectable) to comply with clean room standards.
It can be installed within a Class D to B laboratory (particle emissions are minimal, as it does not use liquid N_2) . **IQ/OQ/PQ (installation, operation, and performance qualification)** services are provided to facilitate its validation in a GMP process.

- **Safety and compatibility:**

The design ensures that magnetic/RF fields are contained primarily within the camera.
Designed to comply with electromagnetic compatibility regulations (e.g. monitors, incubators) in the same room.
Safety interlock doors prevent accidental openings during a cycle.
It includes visual/audible and historical alarms for temperature deviations or failures, with the possibility of remote monitoring.

- **Maintenance:**

annual preventative maintenance : inspection of the compressor and refrigeration circuit, and verification of the calibration of the temperature and electromagnetic field strength sensors. The permanent magnets do not lose significant power over time, so they do not require routine replacement. Software and firmware updates can be performed on-site or remotely by authorized technicians. The estimated lifespan of the equipment exceeds 10 years of continuous use, thanks to its lack of extreme cryogenics, its robust design (many conventional ultra-low temperature freezers fail around 10 years), and the fact that 100% of its components are manufactured in Japan (Mitsubishi brand).

- **Regulatory status:**

The PF-15 NEO is currently marketed as **laboratory equipment** (not a medical device), with CE marking under the Machinery Directive. The manufacturer is pursuing additional certifications in accordance with local regulations to facilitate its clinical use (e.g., in Europe it could eventually be classified as a Class I device for cell processing). (See *Regulatory Framework section* below.)

- The equipment **does not come into direct contact** with the patient nor does it constitute an **implantable medical device** , but rather process equipment within the manufacturing chain.

PROTON NEO as a technological infrastructure for the cell therapy industry

The industrialization of iPS-derived cell therapies implies a structural change in the manufacturing chain.
Cryopreservation ceases to be an auxiliary laboratory phase and becomes a **critical process infrastructure** within the GMP system.
In this context, PROTON NEO should be understood not as a conventional freezer, but as a **technological platform for structural control of freezing** , with direct implications for quality, reproducibility, and scalability.

1 - Structural control of crystallization

In conventional freezing systems, ice nucleation occurs heterogeneously and partially randomly.
This generates:

- Crystals of variable size
- Local thermal gradients
- Irregular osmotic tensions
- Membrane and cytoskeleton damage

PROTON NEO introduces a differentiating element:

- uniform static magnetic field
- Electromagnetic modulation during cooling

The result is not simply "freezing faster", but **conditioning the formation of crystals** .
From a physical point of view:

- The formation of multiple crystallization nuclei is favored.
- The probability of macrocrystal growth is reduced.
- The intracellular and extracellular ice structure is homogenized.

In a cellular environment (iPS cells, dopaminergic progenitors, MSCs, etc.), this directly impacts:

- Plasma membrane integrity
- Conservation of phenotypic markers
- Post-thaw mitochondrial activity
- Functional recovery

Therefore, the system acts on a **structural variable of the process** , not only on the temperature.

2- Electromagnetic modulation as a process parameter

In GMP processes, every critical step must be:

- Controlled
- Programmable
- Reproducible
- Documentable

PROTON NEO allows:

- Programming of defined thermal profiles
- Electromagnetic field intensity control
- Digital recording of cycle parameters
- Exact repetition of validated conditions

This makes electromagnetic modulation a **controllable process parameter (Process Parameter)** .
From a regulatory perspective:

- It can be defined as CPP (Critical Process) Parameter) if the validation proves it.
- It can be incorporated into the process validation dossier.
- It allows batch-to-batch traceability.

This point is key to its positioning in industrial manufacturing.

3- Batch reproducibility

The transition from clinical trial to commercialization requires:

- Inter-batch homogeneity
- Consistency of post-thaw viability
- Phenotype stability
- Predictability of functional recovery

Freezing is one of the phases that introduces the greatest variability if it is not structurally controlled.
PROTON NEO provides:

- Uniformity of forced airflow
- Volume-controlled chamber
- Homogeneous distribution of electromagnetic field
- Load capacity defined by standardized trays

This allows:

- Validation of reproducible thermal curves
- Reduction of deviations between batches
- Establishment of post-thawing acceptance criteria

In a master cell bank (MCB) or working cell bank (WCB), the reproducibility of freezing is crucial for the long-term stability of the product.

4-Applicability in CDMO (Contract Development and Manufacturing Organizations)

CDMOs dedicated to cell therapies require :

- Scalable systems
- Transferable protocols
- IQ/OQ/PQ Validation
- Integration into classified rooms
- Documentation compliant with regulatory audits

PROTON NEO has features compatible with this environment:

- Sanitary stainless steel construction
- Absence of liquid cryogenics (reduces risks and structural requirements)
- Programmable digital control
- Standard industrial electricity consumption
- Easy integration into GMP layout

Besides:
The nominal capacity (~15 kg/h) allows processing relevant volumes for:

- Clinical production
- Initial commercial batches
- Expansion of cell phone banks

This positions the system as an intermediate platform between research laboratory and initial industrial production.

5- Integration into master cell banks (MCB / WCB)

In the manufacture of cell therapies, the master cell bank constitutes the origin of all subsequent production.
Critical requirements:

- Genetic stability
- Phenotypic conservation
- Complete traceability
- Minimal freezing-induced variability

PROTON NEO can be integrated into:

- MCB Generation
- WCB Creation
- Controlled re-expansion
- Preparation of clinical batches

By controlling crystallization and reducing cellular structural damage, the system potentially contributes to:

- Maintaining cellular identity
- Reduce post-thaw variability
- Minimize functional losses

This has direct implications for:

- Robustness of the process
- Product safety
- Therapeutic consistency

6- Paradigm shift: from auxiliary equipment to critical infrastructure

Regulatory approval of iPS therapies in Japan marks the beginning of an industrial phase in regenerative medicine.
In this phase:

- Cryopreservation is not a secondary technical step.
- It is a structural component of the manufacturing system.

PROTON NEO is positioned as:

- Functional freezing platform
- Structural ice control system
- Process reproducibility tool
- Technological infrastructure that can be integrated into GMP

Taken together, these specifications highlight that the PROTON PF-15 NEO is a **highly specialized piece of equipment** that offers precise control of the freezing process at biomedical production scale, while maintaining compatibility with GMP environments and operational safety.

Its incorporation into cell therapy laboratories allows for the standardization and scaling up of functional cryopreservation with minimal additional validation effort, since the manufacturer provides performance documentation and specific technical support.

7- Industrial relevance of PROTON *PF-15 NEO* in iPS cell therapies

1- Transition to commercial phase

The recent approval in Japan of iPS-derived products for Parkinson's disease (AMCHEPRY® – Sumitomo Pharma) marks the beginning of the first global commercialization of allogeneic iPS cell therapies.

2- Critical quality variable

In the context of industrial manufacturing of cell therapies, cryopreservation ceases to be an experimental procedure and becomes a critical quality variable (Critical Process) . Parameter , CPP).

3- Technical positioning

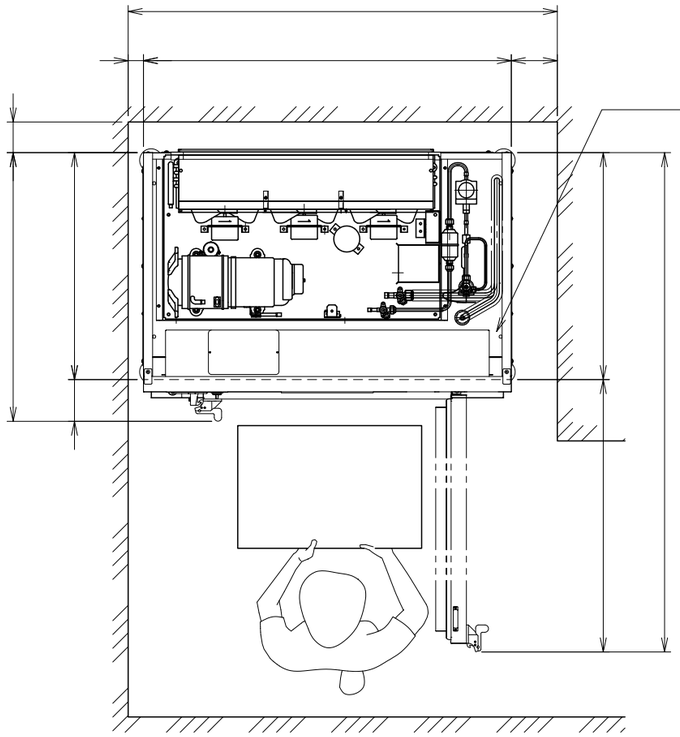
PROTON technology has been used in scientific studies linked to the development of iPS-derived dopaminergic neurospheres relevant to Parkinson's clinical programs, demonstrating maintenance of dopaminergic markers and post-thaw functionality.

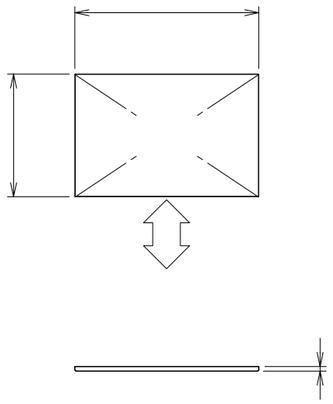
P F - 1 5 NEO SPECIFICATION

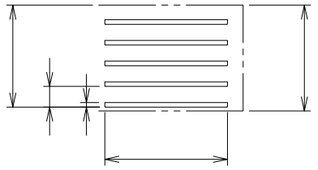
item	specification
standard specification Usage	Quick Freezing Equipment for Foods
Internal temperature setting	Air blast method -30° C to -35° C
Incoming product temperature	The standard temperature for entering the warehouse is about
	20° C → -18° C or lower.
Throughput	Nominal 15kg/h ※60Hz operation.
	Capable of freezing 15 kg of food per hour.
	The moisture content in the food is assumed to be 50% and
	based on in-house load calculations.
Freezing time	Freezing time varies greatly depending on size, loading conditions,
	and physical properties.Please confirm the guideline in prior testing.
Approximate capacity	About 5～ 7 trays of 600 x 400 can be stored:
	1 shelf specification x 1 unit.
	Maximum storage height is 350mm.
External Dimensions	Please check the outline drawing.
Tray Size	600W x 400D x 15H (dedicated tray)
Transmitter Unit	Transmitting amplifier x 1, Antenna x 2
Number of yoke unit mechanism sets	Nominal “600W x 400D x 350H” x 1 horizontal row x 1 unit
	Neodymium Special Magnet Unit
Body Structure	The surface material inside and outside the chamber is SUS304.
	Urethane is used for insulation.
Refrigeration unit	3HP, R404A, air-cooled rotary condensing unit
Cooling Fan	Slim Pressure-Balanced Ventilation Fan <Stainless Steel Type>
	※Regular replacement is required depending on the operating
	environment in low temperature range.
Operation and display system	Display and operation by GOT touch panel.
Cooling unit	Copper tube aluminum fin + MHAT21 coating, defrost should be
	cleaned manually.
Continuous Operation Time	Approx. 8 hours *Depends on the installation environment
	and frequency of opening/closing.
Defrost system	Please manually clean the hose from the top of the heat
	exchanger every day.
Main unit weight	Approx. 400kg
Power Specifications	
Power Specifications	Three-phase AC200V 50/60Hz
Power Consumption	３．４ｋW(50Hz)／３．９ｋW(60Hz)
Rated current	１６．２Ａ(50Hz)／１６．１５Ａ(60Hz)
Circuit Breaker Capacity ２０Ａ	２０Ａ
Outlet Shape	Power cable 3m with waterproof cap
	(Grounding 3P/250V/20A)

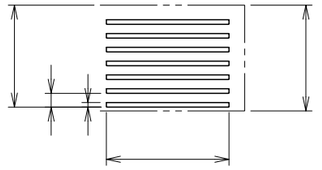
proton

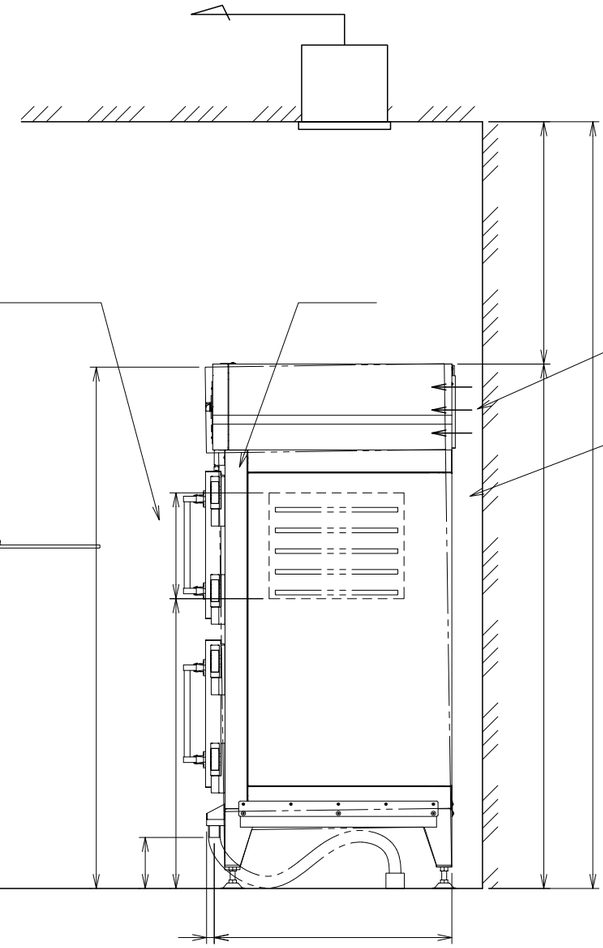
RB

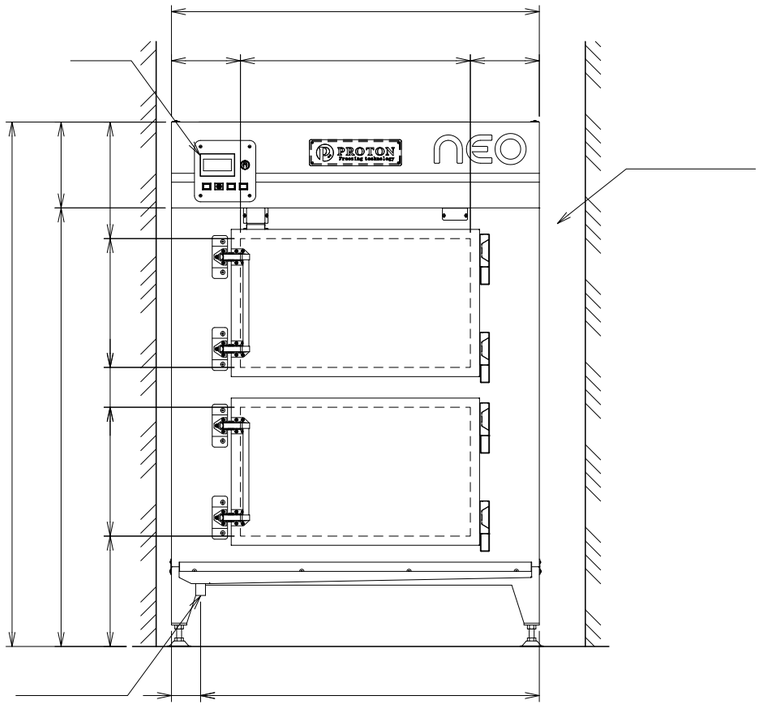












Annex D – References to cited academic publications

(Complete bibliographic references for the key citations used in the report are listed below for further consultation by the interested reader.)

1. Hiramatsu, S., *et al* . (2022). **Cryopreservation of Induced Pluripotent Stem Cell-Derived Dopaminergic Neurospheres for Clinical Application** , *Journal of Parkinson's Disease, 12* (3), 871–884. DOI: 10.3233/JPD-212934. – *Demonstrates the effectiveness of PROTON Freezer in preserving dopaminergic neurospheres and their functionality in vitro/in vivo.*
   SEE ANNEX A-1

2. Ayllón Rived, M. (2025). **Scientific Validation of PROTON Magnetic Freezing Technology for Biomedical Cryopreservation** , *J. of Biomedical Advanced Science & Research, 1* (1), 1–10. – *Multi-model review/study confirming the superiority of PROTON in viability and reduction of damage in neuronal, stem, and embryonic cells.*
   SEE ANNEX A-2

3. Patent JP2025094837A (2022). **「細胞の凍結方法」 (Method for freezing cells)** . – *Japanese patent describing the PROTON method; includes experimental examples of freezing iPSC neurons with electromagnetic fields.*
   SEE ANNEX A-3

4. Patent EP4063496A1 (2022). **"Cell Cryopreservation Method and Program (PROTON)"** . – *European patent application detailing the cryopreservation protocol for neurospheres under EM fields and controlled cooling.*
   SEE ANNEX A-4

5. **NIBIOHN – Ryoho Freeze Systems – Chubu Electric Power (May 30, 2023).**
   "Joint research on cell cryopreservation for future medicine" **– Joint official statement announcing the start of collaborative research on cell cryopreservation applied to regenerative medicine and drug discovery. The document describes the validation of PROTON equipment for cell products with preserved nerve function, the optimization of freezing conditions for different cell types (including spheroids and organoids), and the development of specific equipment for biomedical applications. It includes a roadmap for basic research (2 years) and a subsequent validation and implementation phase.**

(Other internal references and technical documents were used to prepare this report, including comparative patent analyses and materials provided by PROTON Europe. For further information or access to these documents, please contact PROTON Europe SL.)

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Press Release

May 30, 2023 National

Institutes of Biomedical Innovation, Health and Nutrition Ryoho

Freeze Systems Co., Ltd. Chubu Electric

Power Co., Inc.

Started joint research on cryopreservation of cells for future medical care

National Institutes of Biomedical Innovation, Health and Nutrition (Chairman: Yusuke Nakamura, Location: Ibaraki City, Osaka Prefecture, hereinafter referred to as “NIBIOHN”), Ryoho Freeze Systems Co., Ltd. (Representative Director: Kazunari Ninomiya) , location: Nara City, Nara Prefecture; We have concluded a joint research agreement on cell cryopreservation in the field of regenerative medicine and drug discovery, and have decided to start joint research.

[Background] Regenerative medicine uses cells, tissues, etc.* (hereinafter referred to as "cell products") to It has the potential to cure difficult-to-treat diseases, and is expected to become a future medical treatment.

In order to make regenerative medicine more familiar, it is essential to develop technology to cryopreserve cell products while retaining their original functions.

[Efforts by the three parties so far] ÿ Ryoho Freeze and Chubu Electric Power have developed technology to freeze food while preserving its deliciousness. We have been doing research and development.

ÿ NIBIOHN has also made it possible to cryopreserve cell products with neural functions, which had been difficult in the past, without impairing their functions by using Ryoho Freeze freezers.

[Significance of this research] Cell products used for treatment differ depending on the type of disease, and finding the optimal freezing conditions that maintain a viable state for various cell products after thawing will be important in realizing future medical care. is a big issue.

Therefore, the three parties will combine the knowledge they have cultivated so far to optimize the freezing conditions for cell products and develop a freezer that is easier to use. We aim to jointly develop technologies and contribute to the development of regenerative medicine and drug discovery.

* Aggregates of cells, spheroids (aggregates of a single species), organoids (aggregates of multiple species), and cells Sheet-like or clump-like cells that take an intermediate state between tissues.

Attachment: Overview of joint research on cell cryopreservation

that's all

<Contact information>

National Institute of Medical Innovation, Health and Nutrition Strategy PR Team +81-72-641-9832 Ryoho Freeze Systems Co., Ltd. Sales Department +81-742-36-9056) 961-3582

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Overview of joint research on cell cryopreservation

May 30, 2023

National Institute of Biomedical Innovation, Health and Nutrition Ryoho Freeze Systems Co., Ltd.
Chubu Electric Power Co., Inc.

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Current status and issues of cryopreservation technology

Food field		Medical (bio) field
Ingredients such as meat and fish are frozen and distributed	current situation	Cell products are difficult to freeze, cancer cell lines (for research) are cryopreserved and distributed
Research and development of technology for deliciously freezing foods (sushi, cakes, etc.)	Recent trends	Research and development of freezing technology for cell products (cornea, skin, etc.)
Freezes and thaws food to maintain raw texture, additive-free storage	Future challenges	Freezing and stocking cell products used for transplantation to realize regenerative medicine

Cross-sectoral development of new technology

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R&D to date

Freezer development in the food field (Ryoho Freeze Systems x Chubu Electric Power)

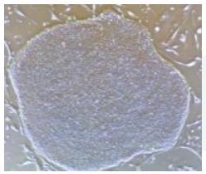
freezing machine

"Proton Neo"

Development of freezing technology in the medical (bio) field (Pharmaceutical Innovation, Health and Nutrition Research Institute x Ryoho Freeze Systems)

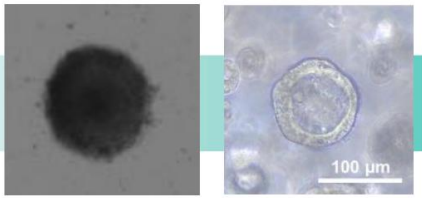
医療分野の
細胞・組織凍結用に
凍結機を小型化

material



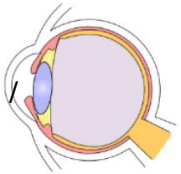
Differentiation induction

intermediate product

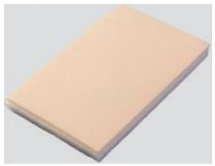


Cryopreserved
Thaw/Culture

Regenerative medicine final products



skin sheet



iPS cells, etc.

Spheroid Organoid

•Intermediate products (spheroids and organoids) can be cryopreserved•Final products used in regenerative medicine can be cryopreserved and are expected to be ready for use when desired

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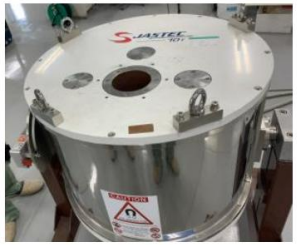
Contents of joint research *From May 2023

Development of easier-to-use freezing equipment

Adopting advanced technology such as superconducting technology in freezing equipment, developing freezing equipment to explore optimal conditions

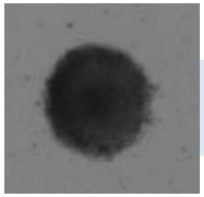


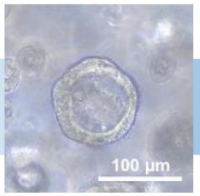
Superconducting device

Development machine image

Revalidation of freezing technology and optimization of freezing conditions

ÿ Re-examine previously successful freezing of cells, etc. ÿ Optimize freezing conditions using the developed equipment





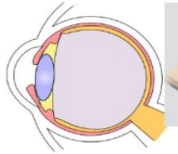
Spheroid Organoid ÿ

Cryopreservation/thawing/cultivation

regenerative medicine final product

Development of regenerative medicine and drug discovery using freezing technology

•Technological development for cell cryopreservation to support future regenerative medicine •Exploring and proposing suitable freezing conditions for each cell product



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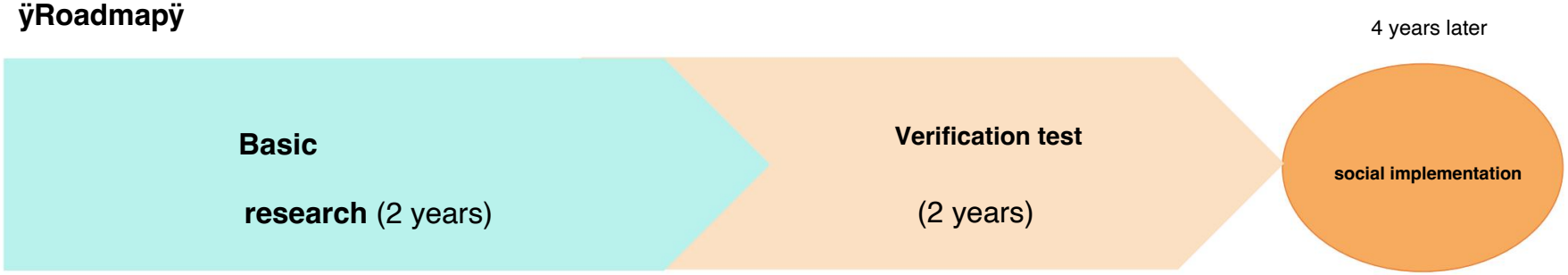
Division of roles for the three parties

Joint research items	NIBIOHN	Ryoho Freeze	Chubu Electric Power
Creation of cell test method, evaluation	ÿ		ÿ
Provision of cells and protective agents	ÿ		
Freeze/thaw test	ÿ		ÿ
Freezer design and production		ÿ	ÿ

ÿRoadmapÿ



Basic research is scheduled for two years. After that, after verification test

We are aiming for social implementation (dissemination of freezing machines/demonstration of freezing of regenerative medicine products) in four years.

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Application range/future prospects

Evolution of cell freezing technology and its potential to contribute to future medical care

1 It was difficult to freeze masses such as tissues and return them to their original state after thawing.

2 It became clear that spheroids can be cryopreserved, and by developing a freezer that is easier to use and optimizing the freezing conditions, frozen stocks of cell products that can be used in regenerative medicine become possible.

3 Make it easy to get the products you need, when you need them

Significant cost cuts are possible by freezing and preserving mass-produced products

There is a possibility that it will develop into organ freezing for organ transplantation, and we will contribute to the development of regenerative medicine and drug discovery that will support future medical care.

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Press Release

30 de mayo de 2023

Institutos Nacionales de Innovación Biomédica, Salud y Nutrición Ryoho
Freeze Systems Co., Ltd. Chubu Electric Power Co.,
Inc.

"Inicia una investigación conjunta sobre criopreservación celular orientada a la atención médica del futuro"

Institutos Nacionales de Innovación Biomédica, Salud y Nutrición (Presidente: Yusuke Nakamura, Ubicación: Ciudad de Ibaraki, Prefectura de Osaka, en lo sucesivo denominado “NIBIOHN”), Ryoho Freeze Systems Co., Ltd. (Director Representante: Kazunari Ninomiya), ubicación: Ciudad de Nara, Prefectura de Nara; Hemos firmado un acuerdo de investigación conjunta sobre criopreservación celular en el campo de la medicina regenerativa y el descubrimiento de fármacos, y hemos decidido iniciar una investigación conjunta.

[Antecedentes] La medicina regenerativa utiliza células, tejidos, etc.* (en lo sucesivo, "productos celulares") para Tiene el potencial de curar enfermedades difíciles de tratar y se espera que se convierta en un tratamiento médico en el futuro.

Para hacer más accesible la medicina regenerativa, es esencial desarrollar tecnología para criopreservar productos celulares conservando sus funciones originales.

[Esfuerzos de las tres partes hasta ahora] Ryoho Freeze y Chubu Electric Power han desarrollado tecnología para congelar alimentos conservando su delicia. Hemos estado haciendo investigación y desarrollo.

NIBIOHN también ha hecho posible criopreservar productos celulares con funciones neuronales, lo que había sido difícil en el pasado, sin perjudicar sus funciones mediante el uso de congeladores Ryoho Freeze.

[Importancia de esta investigación] Los productos celulares utilizados para el tratamiento difieren según el tipo de enfermedad, y encontrar las condiciones de congelación óptimas que mantengan la viabilidad de varios productos celulares después de la descongelación será importante para lograr la atención médica futura.

Por lo tanto, las tres partes combinarán el conocimiento que cada uno ha cultivado para optimizar las condiciones de congelación de los productos celulares y desarrollar una máquina de congelación más fácil de usar, logrando así la congelación necesaria para realizar la atención médica futura. contribuir al desarrollo de la medicina regenerativa y al descubrimiento de fármacos.

* Agregados de células, esferoides (agregados de una sola especie), organoides (agregados de múltiples especies) y células. Células en forma de láminas o grupos que adoptan un estado intermedio de tejido.

Anexo: Descripción general de la investigación conjunta sobre criopreservación celular

eso es todo

<Consultas>

Equipo de relaciones públicas del Instituto Nacional de Innovación Médica, Estrategia de Salud y Nutrición +81-72-641-9832

Departamento de ventas de Ryoho Freeze Systems Co., Ltd. +81-742-36-9056) 961-3582

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Adjunto

Resumen de la investigación conjunta sobre criopreservación celular

30 de mayo de 2023

Instituto Nacional de Innovación Biomédica, Salud y Nutrición Ryoho Freeze Systems Co., Ltd.
Chubu Electric Power Co., Inc.

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Estado actual y problemas de la tecnología de criopreservación

campo de alimentos		Campo médico (bio)
Ingredientes como carne y pescado se congelan y distribuyen	situación actual	Los productos celulares son difíciles de congelar, las líneas celulares cancerosas (para investigación) se criopreservan y distribuyen
Investigación y desarrollo de tecnología para congelar deliciosamente alimentos (sushi, tartas, etc.)	Tendencias recientes	Investigación y desarrollo de tecnología de congelación de productos celulares (córnea, piel, etc.)
Congela y descongela alimentos para mantener la textura cruda y almacenamiento sin aditivos.	desafíos futuros	Congelar y almacenar productos celulares utilizados para trasplantes para realizar medicina regenerativa

Desarrollo intersectorial de nuevas tecnologías.

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I+D hasta la fecha

Desarrollo de congeladores en el sector alimentario (Ryoho Freeze Systems x Chubu Electric Power)

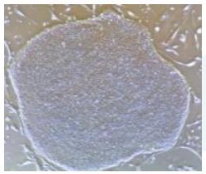
máquina de congelación

"Protón Neo"

Desarrollo de tecnología de congelación en el campo médico (bio) (Instituto de Investigación de Innovación Farmacéutica, Salud y Nutrición x Ryoho Freeze Systems)

医療分野の
細胞・組織凍結用に
凍結機を小型化

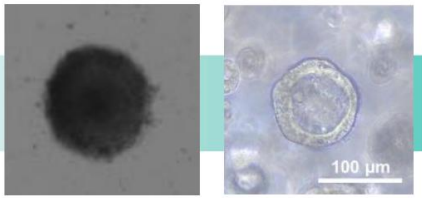
material



Células IPS, etc.

inducción de diferenciación

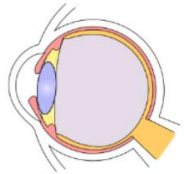
producto intermedio



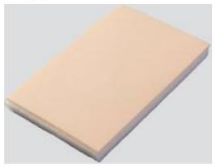
Organoide esferoide

Córnea criopreservada /
descongelación/cultivo

Producto final de la medicina regenerativa



hoja
de piel



•Los productos intermedios (esferoides y organoides) se pueden criopreservar.•Los productos finales utilizados en medicina regenerativa se pueden criopreservar y se espera que puedan usarse inmediatamente cuando se desee.

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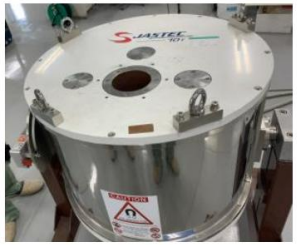
Contenidos de la investigación conjunta

*A partir de mayo de 2023

Desarrollo de equipos de congelación más fáciles de usar.

Adoptar tecnología avanzada, como la tecnología superconductora en equipos de congelación, desarrollar equipos de congelación para explorar condiciones óptimas.

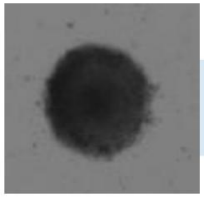


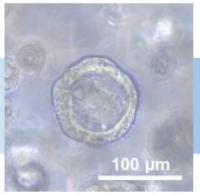
Dispositivo superconductor

Imagen de la máquina de desarrollo

Reexamen de la tecnología de congelación y optimización de las condiciones de congelación.

(1) Reexamen de la congelación exitosa de células, etc. (2) Optimización de las condiciones de congelación utilizando equipos desarrollados





Organoide esferoide

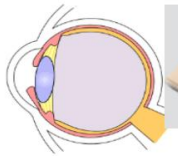
Criopreservación/descongelación/cultivo

producto final de medicina regenerativa

Desarrollo de la medicina regenerativa y descubrimiento de fármacos mediante tecnología de congelación.

•Desarrollo tecnológico para la criopreservación celular para apoyar la futura medicina regenerativa

•Explorar y proponer condiciones de congelación adecuadas para cada producto celular



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División de roles para las tres partes

Artículos de investigación conjunta	NIBIOHN	ryoho Congelar	Energía Eléctrica Chubu
Creación de método de prueba celular, evaluación			
Proporcionando células y agentes protectores.			
Prueba de congelación/descongelación			
Diseño y fabricación de congeladores.			

Hoja de ruta

La investigación básica está prevista para dos años. Después de eso, después de la prueba de verificación.

Nuestro objetivo es lograr una implementación social (divulgación de máquinas de congelación/demostración de congelación de productos de medicina regenerativa) en cuatro años.

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Ámbito de aplicación y perspectivas de futuro

Evolución de la tecnología de congelación celular y posibilidad de contribución a la medicina del futuro

1 Era difícil congelar masas como tejidos y devolverlas a su estado original después de descongelarlas.

2 Quedó claro que los esferoides se pueden criopreservar y, al desarrollar un congelador que sea más fácil de usar y optimizar las condiciones de congelación, se hacen posibles reservas congeladas de productos celulares que pueden usarse en medicina regenerativa.

3 Facilite la obtención de los productos que necesita, cuando los necesita

Es posible una reducción significativa de costos mediante la congelación y conservación de productos producidos en masa.

Existe la posibilidad de que se convierta en la congelación de órganos para trasplantes de órganos, y contribuiremos al desarrollo de la medicina regenerativa y al descubrimiento de fármacos que respaldarán la atención médica futura.

Press Release

2023 年 5 月 30 日
国立研究開発法人　医薬基盤・健康・栄養研究所
株式会社菱豊フリーズシステムズ
中部電力株式会社

「未来の医療を目指した細胞凍結保存に関する共同研究」を開始

国立研究開発法人 医薬基盤・健康・栄養研究所（理事長：中村祐輔、所在地：大阪府茨木市、以下「NIBIOHN（ニビオン）」という。）、株式会社菱豊フリーズシステムズ（代表取締役：二宮一就、所在地：奈良県奈良市、以下「菱豊フリーズ」という。）および中部電力株式会社（代表取締役社長：林欣吾、所在地：愛知県名古屋市、以下「中部電力」という。）は、このたび、再生医療・創薬の分野における細胞凍結保存の共同研究契約を締結し、共同研究を開始することといたしました。

【背景】
再生医療は、細胞・組織・その他※等（以下「細胞製品」）を用いることにより、これまで治療困難であった病気を治す可能性があり、未来の医療として期待されています。
再生医療をより身近な医療にするためには、本来の機能を有した状態で細胞製品を凍結保存する技術の開発が欠かせません。

【これまでの三者の取組】
㋐ 菱豊フリーズと中部電力は、これまで食材の美味しさを保ちながら冷凍する技術の研究開発を進めてきました。
㋑ NIBIOHN も、菱豊フリーズ製の凍結機を使用することで、従来困難であった神経の機能を有した細胞製品をその機能を損なうことなく凍結保存することを可能にしました。

【本研究の意義】
病気の種類によって治療に用いる細胞製品は異なり、様々な細胞製品について解凍後も生きた状態を維持する最適な凍結条件を見つけることが、今後未来の医療を実現する上での大きな課題です。
そこで、三者は、これまでそれぞれが培ってきた知見を組み合わせて、細胞製品の凍結条件の最適化や、より利用しやすい凍結機の開発に取り組み、未来の医療を実現するために必要な凍結技術を共同で開発し、再生医療・創薬の発展に貢献することを目指します。

※ 細胞の集合体であるスフェロイド（単一種の集合体）、オルガノイド（複数種の集合体）、細胞と組織の中間的な状態をとるシート状・かたまり状の細胞等。

別紙：細胞凍結保存に関する共同研究概要

以　上

＜お問い合わせ先＞
国立研究開発法人医療基盤・健康・栄養研究所　戦略 PR チーム（072）641-9832
株式会社菱豊フリーズシステムズ　営業部　（0742）36-9056
中部電力株式会社 総務・広報・地域共生本部　報道グループ　（052）961-3582

別　紙

細胞凍結保存に関する
共同研究概要

2023年5月30日

国立研究開発法人 医薬基盤・健康・栄養研究所
株式会社菱豊フリーズシステムズ
中部電力株式会社

凍結保存技術の現状と課題

食品分野		医療（バイオ）分野
肉・魚など食材は凍結保存されて流通	現状	細胞製品は凍結困難、がん細胞株（研究用）などは凍結保存されて流通
食品（寿司、ケーキなど）をおいしく凍結する技術を研究開発	近年の動向	細胞製品（角膜、皮膚など）の凍結技術を研究開発
食品を凍結・解凍し生の食感を維持、無添加保存	将来の課題	再生医療を実現するため移植に用いる細胞製品を凍結してストックする

分野横断による新技術の開発

これまでの研究開発

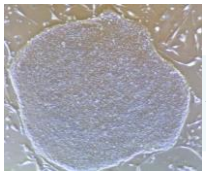
食品分野での凍結機開発（菱豊フリーズシステムズ×中部電力）

凍結機
「Proton Neo（プロトンネオ）」

医療（バイオ）分野での凍結技術開発（医薬基盤・健康・栄養研究所×菱豊フリーズシステムズ）

医療分野の
細胞・組織凍結用に
凍結機を小型化

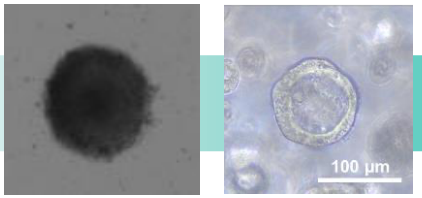
材料



iPS細胞など

分化誘導

中間製品

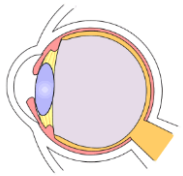


スフェロイド　オルガノイド

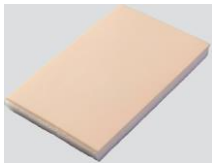
凍結保存
/解凍・培養

再生医療最終製品

角膜



皮膚
シート



・中間製品（スフェロイド・オルガノイド）については凍結保存可能
・再生医療に用いる最終製品を凍結保存が可能で、使いたい時にすぐに使えるようになることが期待されている

共同研究の内容　※2023年5月から

より使いやすい凍結機器の開発

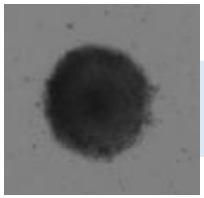
超電導技術等の高度技術も凍結機器に採用し、
最適化条件を探るための凍結機器を開発

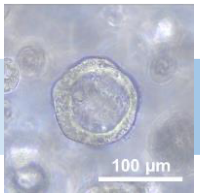
超電導装置

開発機イメージ

凍結技術の再検証と凍結条件の最適化

①これまでに成功している細胞等の凍結を再検証
②開発した機器によって凍結条件を最適化する


スフェロイド



オルガノイド

凍結保存/解凍・培養

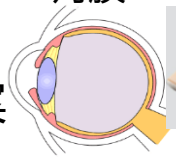
再生医療
最終製品

①

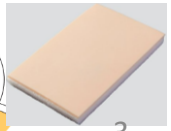
凍結技術による再生医療、創薬の発展

・未来の再生医療を支える細胞凍結保存に技術展開
・それぞれの細胞製品に適した凍結条件を探索・提案

角膜



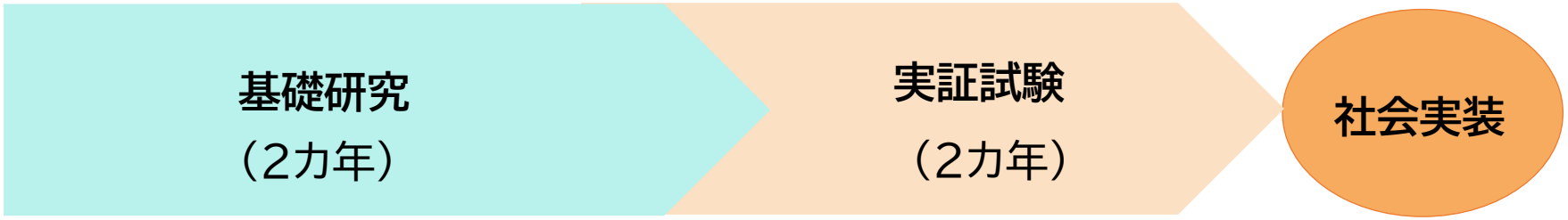
皮膚シート



3者の役割分担

共同研究の項目	NIBIOHN	菱豊フリーズ	中部電力
細胞試験方法作成、評価	○		○
細胞・保護剤提供	○		
凍結・解凍試験	○		○
凍結機の設計・製作		○	○

～ロードマップ～



基礎研究は2カ年を予定。その後、実証試験を経て
社会実装（凍結機の普及/再生医療等製品の凍結実証）は4年後を目指しております。

応用範囲・将来の展望

細胞凍結技術の進化と未来の医療への貢献の可能性

1 組織などの塊りを凍結し、解凍後に本来の機能を有した状態に戻すことが困難であった

2 スフェロイドで凍結保存することが可能であることが明らかとなり、より利用しやすい凍結機の開発、凍結条件の最適化を行うことで再生医療を実現する細胞製品の凍結ストックが可能に

3 必要な時に必要な製品を容易に入手できるように大量に生産したものを凍結保存しておくことで大幅なコストカットが可能に

臓器移植の臓器凍結などに発展する可能性があり、未来の医療を支える再生医療、創薬の発展に貢献します

Annex E – Biomedical Validation Protocol (Example for implementation)

Aim:

To comparatively test the effectiveness of PROTON technology against traditional freezing methods in the preservation of a cell line of biomedical interest, evaluating viability, functionality and post-thaw morphology under controlled conditions.

Cell phone model:

cryosensitive cell population .
For example, **human mesenchymal stem cells (hMSCs)** derived from bone marrow or adipose tissue, or **dopaminergic neurospheres derived from iPSCs** (3D, ~200–300 µm).
These representative cell therapies (tissue regeneration or Parkinson's, respectively) will allow us to measure differences in survival and function after cryopreservation.

Cryoprotective medium:

Use a standard clinical grade cryoprotectant, e.g. **Bambanker hRM** (contains ~10% DMSO) or other GMP medium with 5–10% DMSO + serum/albumin.
Prepare the cells in suspension or aggregates in this medium at 4 °C before freezing (incubate ~10 min to equilibrate; in neurospheres, ensure homogeneous immersion in the CPA).

Rates and format:

Dispense the cells into sterile 1.8 mL cryovials , with a typical volume of 1 mL per vial (containing e.g. 1–5 × 10^6 cells/vial for hMSC , or ~20 neurospheres/vial).
Use at least *N* = 5 vials per condition for statistical robustness.

Conditions to compare:

- **Condition 1 – Slow freezing -80°C:**

Place vials in a slow cooling chamber (e.g., a “Mr. Frosty ” type isopropanol container) to achieve ~ -1 °C/min temperature drop. Start at room temperature down to -80 °C (this takes ~80 min).
After ~4 hours at -80 °C, transfer the vials to storage in the same freezer at -80 °C (if the test is evaluated in days) or LN $_2$ if prolonged storage is intended (>1 month).
This condition reflects a simple conventional protocol (without nucleation control).

- **Condition 2 – Controlled freezing with LN_2 :**

Use a programmable **rate-controlled freezer** (if available) or a manual protocol combined with LN_2 .
Example: cool to -1 °C/min from 4 °C down to -40 °C, then increase the speed to -5 °C/min down to -100 °C.
Apply manually induced nucleation around -5 to -10 °C (ice seed) to simulate best practices.
After reaching -100 °C, **transfer the vials to liquid nitrogen** (vapor phase, ≤ -150 °C) for storage at least overnight.
This condition represents the traditional *gold standard* (scheduled freezing + LN_2) as a positive control of better conventional conditions.

- **Condition 3 – PROTON magnetic freezing:**

Use the **PROTON PF-15 NEO equipment** .
Place the vials in the pre-cooled freezing chamber (~4 °C).
Program a cooling profile: **gradual decrease ~3 °C/ min** from ~0 °C down to -35 °C.
Maintain ~-35 °C for 5–10 min to ensure complete solidification.
Throughout the cooling process, activate the **homogeneous static magnetic field** (~≥5 μT) and the **radiofrequency field** (~0.5–1 MHz, ~100–200 V/m) at the optimum level (e.g., "High") to induce multiple nucleation.
Once the program is completed, quickly transfer the vials to cryogenic storage: ideally to LN_2 steam (since PROTON operates down to ~-35 °C, samples should be brought to -150 °C for long-term stability).
Note: This transfer must be done in <~30 seconds to avoid any partial refreezing; alternatively, they can be temporarily placed in an -80°C ultra-low temperature freezer. °C already cold while they are being transferred to LN 2 .

Defrosting and analysis:

After ≥24 hours of storage under their respective conditions, rapidly **thaw** each vial in a 37°C water bath. °C until the last trace of ice disappears (by gently shaking it).
Immediately transfer to a cool, warm medium, gradually removing the CPA (dilution or exchange in 2–3 steps to avoid osmotic shock).

Assess:

- **Cell viability:**

hemocytometer count , and corroborate with a fluorescent assay (e.g. *live/ dead* with Calcein -AM/PI) or automated trypan blue.
Express viability as % of live cells out of total.

- **Functional recovery:**

Depending on the cell type, measure a key attribute after 24–48 h of post- thaw culture .
Examples: for hMSC , evaluate their **clonogenic capacity** in CFU -F (colony formation) and surface markers (CD73 /90/105); for dopaminergic neurons, measure **dopamine release** in the medium in response to a stimulus or record electrical activity/calcium in cultures.
Compare to a control of fresh, unfrozen cells (gold standard = 100% function).

- **Morphology and structure:**

Examine under a light microscope and, if possible, by electron microscopy or immunofluorescence: look for membrane integrity, adherence and normal spar (in adherents), neuritic extension (in neurons, neurite length can be quantified vs control), tissue organization (in aggregates or tissues).
Use viability stains (e.g., FDA/PI) to visualize damaged cells.
Also, specific apoptosis assays (TUNEL, active caspase 3) to quantify sublethally damaged cells in each group.

Success criteria:

Samples frozen with PROTON are expected to exhibit **significantly greater viability** than those frozen at -80 °C and greater than or equal to those of LN 2 .
Likewise, **better functionality** (e.g., differentiation or secretion rates close to fresh control) and **less morphological damage** (fewer rounded cells, with lysed membranes or intracellular vacuolization).
Previous results indicate, for example, that PROTON-frozen hMSCs maintain their proliferative potential almost intact, forming colonies normally after thawing, whereas conventionally frozen hMSCs lose much of that potential.
Similarly, PROTON neurospheres retain extensive neurites (~60% of the length in fresh controls) versus virtually no neurites in frozen neurospheres without PROTON.
These differences must be confirmed statistically (*t test* or ANOVA, p <0.05).

Documentation:

Record the **temperature vs time curves** for each run (PROTON equipment automatically saves this log; for other conditions use probes or program data).
Also note field strengths used, transition times, etc., to correlate with results.
All data obtained (viability, microscopy photos, functional measurements) will be compiled into a comparative report.
This type trial will provide direct experimental evidence under local conditions that PROTON technology **improves cryopreservation** in relevant quantitative and qualitative parameters, serving as a basis to justify its adoption in GMP environments.

Annex F – Institutional recognition and international dissemination

1. NIBIOHN – Ryoho Freeze Systems – Chubu Electric Power (May 30, 2023).
   "Joint research on cell cryopreservation "For Future Medicine" – Joint official statement announcing the start of collaborative research in cell cryopreservation applied to regenerative medicine and drug discovery. The document describes the validation of PROTON equipment for cell products with preserved nerve function, the optimization of freezing conditions for different cell types (including spheroids and organoids), and the development of specific equipment for biomedical applications. It includes a roadmap for basic research (2 years) and a subsequent validation and implementation phase.
   SEE ANNEX D-5

2. Publication in *La Vanguardia* - Title: *" Beyond nitrogen: a before and after in freezing breaks a historical limitation of medicine"*
   Author: Robert Marsà . Date: February 13, 2026
   The article analyzes the impact of the Japanese structural freezing technology PROTON on
   The article highlights the biomedical field, emphasizing that the main challenge in cell cryopreservation is not the final temperature achieved, but rather controlling the crystallization process during the water phase transition. It explains that the uncontrolled formation of ice crystals is the root cause of structural and functional damage in sensitive cells, and that PROTON technology introduces an approach based on the physical control of nucleation using electromagnetic fields, thus reducing the formation of harmful macrocrystals. The article mentions applications in stem cells and iPSCs, including dopaminergic models related to Parkinson's disease, and contextualizes this innovation within the growth of regenerative medicine and advanced cell therapies.

OFRECIDO POR PROTON

PROTON

Más allá del nitrógeno: un antes y un después en la congelación rompe una limitación histórica de la medicina

La tecnología japonesa de congelación magnética PROTON expone un cambio de paradigma en la criopreservación y desbloquea nuevas capacidades terapéuticas

La tecnología de congelación japonesa PROTON ha revolucionado los criterios de congelación para la criopreservación

Robert Marsà

13/02/2026 06:00

Durante décadas, la palabra “congelar” ligada a la ciencia ha escondido el significado de detener el tiempo, de preservar un ecosistema o de establecer una pausa limpia en la degradación natural de un cuerpo. Nada más lejos de la realidad. En el mundo de la criopreservación médica ya se cuenta en más de medio siglo el reinado del nitrógeno líquido y su capacidad para bajar el termómetro a los -196 °C. Como si de una caja fuerte se tratase, el uso de este gas se estableció como la máxima más fiable, como el estándar que garantizaba que lo vivo podía sobrevivir al hielo. Y durante mucho tiempo pareció suficiente.

La congelación mediante la tecnología PROTON respeta la función biológica original de células complejas y tejidos, algo que mediante la congelación por nitrógeno es impensable

Sin embargo, la ciencia no avanza sin preguntas incómodas; ¿y qué más incómodo que cuestionar lo que parecía incuestionable? El nitrógeno ha prevalecido como el principal método de criopreservación por su capacidad de congelación, pero su uso es muy agresivo y expone limitaciones biológicas importantes. Este gas criogénico consumible es infalible a la hora de congelar y, hasta ahora, se ha creído que es el medio perfecto para la criopreservación. Pero una mejora incremental en los criterios de congelación demuestra que el uso del nitrógeno tiene un amplio margen de mejora en su misión.

Lee también

Una tecnología desarrollada en Japón está cambiando cómo se conserva la comida y los tratamientos médicos en todo el mundo

CARLOTA BISBE

El hielo: ¿aliado o enemigo?

Para dar con la tecla, la tecnología japonesa de congelación magnética PROTON viaja hasta el origen de la cuestión. “El problema no es cuán frío se congela, sino cómo se forma el hielo”, sostienen los desarrollos más recientes en este campo. La aplicación del nitrógeno líquido obvia el momento más importante: el de la congelación. Y ahí es cuando muestra sus limitaciones biológicas y PROTON marca la diferencia.

Mediante la congelación por nitrógeno, solo sobreviven entre el 50 y el 70 % de células, mostrando una funcionalidad parcial e inestable tras su descongelación

Para preservar fragmentos de vida, es necesario cuidar el lecho en el que van a descansar. Mediante la congelación por nitrógeno, no existe control en el momento de la congelación, formándose cristales de hielo grandes que perforan membranas celulares, deforman orgánulos y destruyen funciones esenciales. Es como si el hielo, en lugar de proteger, atacara. En este proceso, entre el 80 y el 90% de células no sobreviven, y las que lo hacen, a menudo pierden su funcionalidad.

Las células no solo sobreviven, funcionan

Este caos molecular acaba con la tecnología de congelación magnética PROTON, que en lugar de confiarlo todo a temperaturas extremas, entiende el proceso de congelación desde el control estructural del hielo en el momento de su formación. No congela más, congela mejor. Este cambio de enfoque supone un salto de nivel sustancial en cuanto a la criopreservación, respetando la funcionalidad de las células complejas como las neuronas y reproduciendo entre el 80 y el 90% de ellas en su naturaleza original.

No se trata solo de que la célula siga "viva" al descongelarse, sino de que siga siendo útil. De que mantenga su estructura para así preservar su comportamiento, su capacidad de integrarse en un tejido o de reparar una función dañada. Esta evolución no solo permite preservar neuronas humanas funcionales, sino que cambia el escenario médico y biotecnológico que con el nitrógeno se había planteado.

El uso de la tecnología PROTON en la criopreservación supone un antes y un después en la medicina regenerativa (rawpixel.com / Wit)

Un punto de inflexión que impacta en medicina

La aparición de la tecnología de congelación magnética PROTON no significa la eliminación del nitrógeno de los laboratorios, pero sí que pone fin a la idea simplista

de que este gas criogénico es la máxima de la preservación por congelación. Además, la gran evolución que incorpora en el trato molecular durante el proceso de congelación permite hablar de una nueva capacidad terapéutica. No representa una mejora incremental de la criopreservación existente, representa la aparición de una nueva capacidad clínica: preservar función biológica compleja tras la congelación, algo que los métodos tradicionales basados en nitrógeno no habían logrado hasta ahora.

En la práctica, esto significa poder conservar y almacenar células destinadas a terapias celulares con garantías reales de funcionamiento. Ámbitos como el tratamiento del párkinson se ven beneficiados del trabajo de PROTON. No solo se trata de congelar para guardar, sino de congelar para poder tratar, investigar y aplicarlo a la medicina, que hasta ahora veía en el uso del nitrógeno un impedimento para las técnicas regenerativas.

De los laboratorios a la congelación de alimentos: calidad preservada tras la descongelación

Si una tecnología es capaz de conservar una neurona humana funcional, su aplicación en alimentos se convierte en una demostración casi intuitiva. El mismo principio que evita que el hielo destruya una célula permite que un pescado conserve textura y brillo, que una carne no se degrade o que un plato cocinado mantenga calidad similar a la del producto fresco tras descongelarse.

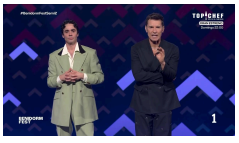
La aplicación de los criterios de congelación de PROTON sobre alimentos supone una mejora incremental de su calidad tras la descongelación

Tras su validación científica y clínica en Japón, la tecnología de congelación magnética PROTON expande ahora su capacidad de preservación de la vida fuera del continente asiático. Viajando a la raíz del problema y cuidando la arquitectura celular en su trabajo, este cambio paradigmático en la criopreservación ya emplea sus medios para revolucionar la medicina.

Más información

Web: **https://proton-europe.com/**

MUNDODEPORTIVO



Todo el mundo señala lo mismo sobre Jesús Vázquez en el Benidorm Fest 2026 de RTVE tras su salida de Telecinco

Annex G – International Coverage of Regulatory Approval of iPS Therapies in Japan (2026)

The following are references to international publications that document the conditional approval by the Japanese Ministry of Health of iPS-derived regenerative medicine products, constituting the first regulated commercialization of this type worldwide.

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1. Bloomberg News (February 19, 2026)
"iPS細胞使った再生医療製品、厚労省専門部会が了承ー世界初の実用化へ"
– Reports the conditional approval by the panel of the Japanese Ministry of Health, Labour and Welfare (MHLW) for two iPS cell-derived products, including the dopaminergic Parkinson's treatment developed by Sumitomo Pharma. – Highlights the historic nature of the first global commercialization of iPS-based therapies.

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2. The Japan Times (2026)
"Japan panel approves stem cell medical products"
– Describes the Japanese regulatory framework that allows conditional approval under efficacy evaluation within a specified timeframe. – Emphasizes the relevance of the Kyoto/CiRA ecosystem in clinical development.

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3. MarketScreener (2026)
"Japan government panel approves 2 iPS-derived drugs globally first"
– Confirms approval under specific Japanese legislation for regenerative medicine. – Indicates Sumitomo Pharma's involvement in the commercialization phase.

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4. CHOSUNBIZ (2026)
"Japan leads stem cell research, advancing Parkinson's treatment with iPS cells"
– Highlights Japan's leadership in pioneering regulation of cell therapies. – Analyzes strategic implications for the global biotechnology industry.

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5. Sumitomo Pharma Official Statement (2026)
"Regarding the Scheduled Review in Japan of the Allogeneic iPS Cell-Derived Dopaminergic Neural Progenitor Cells (AMCHEPRY)"
– Official corporate document confirming review and regulatory process before the PMDA.– Details previous clinical phases and conditional evaluation.

The cited international coverage confirms the transition from clinical research to the regulated commercialization phase of iPS therapies in Japan.
This regulatory context constitutes the environment in which functional cryopreservation acquires the character of critical infrastructure within the cell production chain.

iPS細胞使った再生医療2製品、世界初の実用化へ－心不全とパーキンソン病向け

■ 住友ファーマとクオリプスの2製品、早期承認へ

■ 細胞医療の商業化に大きな節目－市場の利益期待強まり株価急上昇

Komaki Ito、Kanoko Matsuyama

2026年2月19日 at 6:40 GMT+1
更新日時：2026年2月19日 at 12:11 GMT+1

厚生労働省薬事審議会の専門部会は19日、人工多能性幹細胞（iPS細胞）を使った再生医療製品2種について、製造販売の条件・期限付き承認（早期承認）を了承した。同技術を用いた医薬品が実用化されるのは世界初で、日本が成長分野と位置付けてきた細胞医療の商業化が節目を迎える。

了承を受けたのは、大阪大発ベンチャーのクオリプスによる重症心不全向け「リハート」と、住友ファーマのパーキンソン病治療薬「アムシェプリ」。

厚生労働省の発表によると、2社は製造販売について7年間の承認期間を得たうえで、追加データの収集は引き続き行わなければならない。専門部会の了承判断は厚生労働省に送られ、最終的な承認を得る見通しだ。

リハートは、薬や従来の治療法では効果が不十分な虚血性心筋症に対して、iPS細胞から作った心筋細胞シートを心臓の表面に貼り付けることで、血管の再生を促す物質の分泌などを通じ、心機能の改善を目指す。

アムシェプリは、パーキンソン病によりドーパミンが減った患者に、iPS細胞からドーパミン神経前駆細胞を作製し、脳内に移植する。運動機能の改善効果が期待されるという。住友ファーマの木村徹社長は2025年5月、再生・細胞医薬事業について、「30年代の半ばには1000億円と、その後には3500億円の事業にできるように進めていく」と述べていた。

住友化学と住友ファーマは24年、再生・細胞医薬の事業化を加速するため、新会社ラクセラを共同出資（住友化学66.6%、住友ファーマ33.4%）で設立した。

株式市場はすでに承認審査の動きを織り込んでいた。13日にクオリプスと住友ファーマが審議予定だと公表した後、住友ファーマ株は2400円前後から一時3200円台まで上昇。クオリプス株も、9500円前後から1万2000円台まで上昇する場面もあった。日本の効率化された承認システムが、商業的利益につながるという期待を反映している。

シティグループ証券の山口秀丸アナリストは17日付の英文リポートで、報道以降の住友ファーマの株価について「行き過ぎ」だと指摘。ピーク時の年間売上高が1000億円に達し、株価売上高倍率（PSR）4倍で事業価値を評価した場合、評価額は4000億円となる。このうち同社持ち分は約1200億円、1株当たり約300円に相当すると見ていた。

iPS細胞は京都大学の山中伸弥教授が12年にノーベル生理学・医学賞を受賞した技術。日本政府は同技術を成長戦略の柱の1つに据え、1000億円超の研究費を提供してきた。13年には再生医療関連法を整備し、細胞製品の承認審査を迅速化する枠組みを構築。有効性が推定され、安全性が確認された段階で条件・期限付き承認を可能とする制度を導入した。

(最新情報を追加し更新します)

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JAPAN / SCIENCE & HEALTH

Japan succeeds in improving Parkinson's symptoms with iPS cells

Parkinson's disease is a neurodegenerative condition in which motor function is impaired due to a decrease in nerve cells that produce dopamine, a chemical that works in the brain. | GETTY IMAGES

JIJI

Apr 17, 2025

KYOTO – A Japanese group said Thursday that it succeeded in improving symptoms of Parkinson's disease patients with nerve cells produced from induced pluripotent stem (iPS) cells.

Symptoms improved in four of the patients participating in a clinical trial in which nerve cells made from iPS cells were transplanted into their brains, said Kyoto University Hospital and the national university's Center for iPS Cell Research and Application (CiRA).

Sumitomo Pharma, which supplied the nerve cells, plans to apply for a state approval for the treatment under a fast-track system for regenerative medicine products that gives approvals on certain conditions for use over limited periods.

The Osaka-based company aims to gain such an approval within this fiscal year at the earliest. A paper on the clinical trial was published in the British journal Nature.

Parkinson's is a neurodegenerative condition in which motor function is impaired due to a decrease in nerve cells that produce dopamine, a chemical that works in the brain.

Japan is believed to have about 290,000 people with the disease. While treatment of symptoms using drugs is available, no fundamental cure has been established.

Between 2018 and 2023, Kyoto University Hospital and CiRA transplanted 5 million to 10 million dopamine neurons made from iPS cells into the central part of the brains of seven patients between the ages of 50 and 69. The institutes observed their progress for two years to examine the effectiveness and safety.

As a result, no serious side effects were seen in any of the seven. In the six patients checked to see whether the treatment was effective, dopamine nerve activity increased after the transplantation and the amount of dopamine in the brain also rose.

Motor function improved in four of them. Younger patients with milder symptoms tended to show bigger improvements, according to the institutes.

"The treatment has been shown to be effective in patients. It's a great achievement," said Jun Takahashi, a professor at CiRA. "We hope to deliver the cell transplant treatment to many patients as soon as possible."

KEYWORDS

HEALTH(HTTPS://WWW.JAPANTIMES.CO.JP/TAG/HEALTH), MEDICINE(HTTPS://WWW.JAPANTIMES.CO.JP/TAG/MEDICINE), PARKINSON'S (HTTPS://WWW.JAPANTIMES.CO.JP/TAG/PARKINSONS), STEM CELLS(HTTPS://WWW.JAPANTIMES.CO.JP/TAG/STEM-CELLS), IPS (HTTPS://WWW.JAPANTIMES.CO.JP/TAG/IPS)

ACTUALIZACIÓN 2: El panel del gobierno de Japón aprueba dos medicamentos derivados de iPS por primera vez a nivel mundial

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CUORIPS INC. -7,75%

JapónEl panel del Ministerio de Salud aprobó el jueves la comercialización de dos productos de medicina regenerativa preparados a partir de células iPS, los primeros de su tipo en el mundo.

Los dos fármacos desarrollados por ReHeartCuorips Inc., una startup originaria de laUniversidad de Osaka, y Amchepry porCompañía farmacéutica SumitomoyRacthera Inc.Se utilizará en pacientes con insuficiencia cardíaca grave derivada de miocardiopatía isquémica y enfermedad de Parkinson, respectivamente.

"Estoy muy feliz de ver el primer gran paso hacia su implementación social, 20 años después de su anuncio", dijoShinya Yamanaka, director emérito deUniversidad de KiotoCentropara iPSInvestigación celulary aplicación.

Con ReHeart, se colocan láminas de músculo cardíaco derivadas de células iPS humanas en la superficie del corazón, donde promueven la formación de nuevos vasos sanguíneos debajo y ayudan a restaurar la función cardíaca.

A los pacientes con enfermedades cardíacas graves a menudo se les recetan medicamentos para prevenir el deterioro y, a veces, pueden tener que someterse a un trasplante de corazón.

En un ensayo clínico, se trasplantaron láminas de músculo cardíaco a ocho pacientes. El ensayo mostró una reducción de síntomas como el agotamiento y las palpitaciones, así como mejoras en la función cardíaca y la condición física.

Con Amchepry, las células iPS humanas se convierten en "células progenitoras neuronales dopaminérgicas" y se inyectan en el cerebro del paciente.

Las células se trasplantaron a siete pacientes en un ensayo clínico. De los seis pacientes cuya eficacia se evaluó, las células trasplantadas produjeron dopamina, y algunos pacientes también mostraron una mejoría de los síntomas motores.

La enfermedad de Parkinson es una enfermedad neurodegenerativa progresiva causada por una degeneración y pérdida de células cerebrales productoras de dopamina, lo que puede conducir a una disminución de la función motora.

El panel estableció un período de aprobación de siete años, y el producto se lanzará luego de discusiones sobre precio, cobertura de seguro y preparativos para su fabricación y distribución.

Los productos se aprobaron con una capacidad limitada, ya que los estudios clínicos se realizaron en un número reducido de pacientes. Si posteriormente se determina que son seguros, podrán utilizarse plenamente sin restricciones.

Cuorips dijo que su objetivo es comenzar a vender su producto dentro de este año.

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Science

Japan leads stem cell research, advancing Parkinson's treatment with iPS cells

Japan's significant advancements in iPS cell technology pave the way for breakthroughs in treating complex diseases like Parkinson's.

By Heo Ji-yoon

Updated 2025.04.19. 17:35

Illustration=ChatGPT DALL·E 3

In the early 2000s, countries around the world engaged in a research competition over stem cells, dubbed "the flower of future medicine." South Korea got off to a fast start. In 2004, a team led by Dr. Hwang Woo-suk announced that it had successfully cultivated the world's first cloned embryonic stem cells. However, as this paper was revealed to be fabricated, South Korea fell behind, while Japan invested heavily in induced pluripotent stem cells (iPS cells), which eventually earned a Nobel Prize, and moved ahead. This article looks at how the stem cell competition between South Korea and Japan has progressed over the past 20 years.[Editor's note]

The world is paying attention to Japan's succession of research and development (R&D) achievements in stem cell therapies. This is because the "induced pluripotent stem cells (iPS cells)" that Japan has heavily invested in have increased chances of solving intractable diseases such as Parkinson's disease. Analysts suggest that the day of fruition for the research efforts of Japanese scientists, coupled with significant investments from the government and corporations, is imminent.

◇Japan presents the possibility of treating Parkinson's disease

Parkinson's disease is a degenerative disorder in which nerve cells that secrete dopamine in the brain deteriorate, leading to a loss of motor function and tremors throughout the body. Severe cases can also lead to cognitive decline, but there are still no definitive treatments. Japanese scientists have proven that Parkinson's disease can be fundamentally treated using iPS cells.

iPS cells are induced to revert to an embryonic stem cell state capable of growing into any type of human cell by introducing specific genes, proteins, or chemicals into fully developed cells. Embryonic stem cells are obtained from fertilized eggs where sperm and eggs meet, while iPS cells avoid this and can sidestep bioethics controversies. One advantage is that therapies can be created from the patient's own cells, eliminating the need for immunosuppressants.

A research team from Kyoto University published a paper in the international journal Nature on the 17th, detailing the results of Phase 1 and Phase 2 clinical trials conducted on patients with Parkinson's disease. The clinical trials were carried out at

Kyoto University Hospital involving seven patients aged 50 to 69. The iPS cells were differentiated into precursor cells for dopamine nerve cells, which were then transplanted into the brains of the seven patients (three receiving low doses and four receiving high doses) and followed up for 24 months.

The trial results indicated no safety issues. The researchers noted, "No significant side effects appeared to the level of major adverse effects such as tumor formation due to excessive growth of the transplanted cells." A total of 73 mild to moderate adverse reactions were reported. Furthermore, magnetic resonance imaging (MRI) showed no signs of excessive growth of the transplanted cells. Some patients also exhibited a reduction in tremors or rigidity, indicating therapeutic effects.

The Kyoto University research team stated, "We have demonstrated that the homogenous iPSC-derived dopamine precursor cells survive post-administration while producing dopamine, and importantly, do not form tumors," indicating potential safety and clinical efficacy against Parkinson's disease.

Dopamine neural precursor cells made from induced pluripotent stem cells were administered to Parkinson's disease patients. After a 24-month follow-up, no significant

side effects occurred. It is shown to help alleviate Parkinson's disease symptoms. /Nature

◇20 years of research relay… active clinical trials

Kyoto University initiated iPS cell research and is currently leading global research efforts. Professor Shinya Yamanaka of Kyoto University discovered in 2006 that injecting specific genes into mouse skin cells can create iPS cells similar to embryonic stem cells. For this achievement, he received the Nobel Prize in 2012.

With the publication of the latest Nature paper, academia predicts that Japan could become the first nation to officially approve iPS cell treatments for Parkinson's disease. On the same day, Nature commented that "after nearly 20 years of effort and trial and error in the field of stem cells, Japan has reached a "critical point" to introduce a stem cell therapy to market."

Although Japan was the first to open the stem cell therapy market, there is currently no officially approved iPS cell treatment. This is due to the lack of clear therapeutic effects verified in large-scale clinical trials and ongoing concerns about treatment expenses and safety.

Shinya Yamanaka, a professor at Kyoto University in Japan who won the Nobel Prize in Physiology or Medicine in 2012. He pioneered the induced pluripotent stem cell (iPSc) technology that converts mature cells back into the primitive stem cell state. Professor Yamanaka applied to be an assistant professor at Nara Institute of Science and Technology in 1999, and the school hired him despite having no prior accomplishments, as he aimed to pioneer a completely new field. He moved to Kyoto University in 2004 and completed his research. /Kyoto University

Nevertheless, the reason many believe Japan can soon reap the benefits of its investments in stem cell therapy R&D is due to the ongoing advancement of research. According to Nature, over 60 iPS cell therapy clinical trials are currently being conducted worldwide, with about one-third taking place in Japan. The safety of these trials has generally been confirmed, and some have also observed significant therapeutic effects.

Tras su pérdida de peso, la mujer de Pierce Brosnan confirma lo que siempre hemos sabido
Paperela

Since Professor Yamanaka won the Nobel Prize for his research on iPS cells, the Japanese government and academia have focused on investing in this field. Results have followed. In 2014, Dr. Masayo Takahashi from the RIKEN Center successfully transplanted retinal cells derived from iPS cells into patients, marking the world's first.

The transplanted cells survived for more than 10 years, maintaining the patient's vision. However, creating therapies from autologous cells has proven time-consuming and expensive, making commercialization challenging. Dr. Masayo noted to Nature, "We are developing a method to create iPS cell therapies using cells from other individuals," emphasizing that "Japan's regenerative medicine is advancing very rapidly."

Professor Jun Takahashi from Kyoto University, who led the iPS cell treatment research for Parkinson's disease, has transferred the technology to the Japanese pharmaceutical company Sumitomo Pharma for commercialization and is now focusing on developing iPS cell treatments for strokes.

The left photo shows Prime Minister Shinzo Abe visiting the Institute of Biomaterials and Bioengineering together with Professor Shinya Yamanaka of Kyoto University, who won the Nobel Prize in Physiology or Medicine for stem cell research, and Ryoji Noyori, president of the Institute of Biomaterials and Bioengineering and winner of the Nobel Prize in Chemistry in 2001, on January 11, 2013. The right photo shows Crown Prince Naruhito of Japan visiting the Kyoto University iPS Cell Research Institute in 2011, viewing myocardial cells under a microscope. /Chosunilbo

◇Japan's bold investment… rapid approval system introduced

Japanese researchers have been able to continue their research achievements and conduct a "long-distance run" due to a solid policy and institutional foundation.

Since 2013, the Japanese government has decided to invest approximately 110 billion yen (about 1 trillion won) in regenerative medicine research over a period of 10 years. Most of this budget has been used to support research institutions such as Kyoto University's iPS Cell Research Institute. Even after the 10-year investment period ended in 2023, about 9 billion yen (approximately 895 million won) continues to be invested annually.

With the Japanese government providing significant support, large-scale investments from the private sector followed. Scientists are challenging clinical trial research and starting startups. Major biotechnology corporations made substantial investments in manufacturing facilities for cell therapies and opened medical facilities to accommodate patients from Japan and abroad.

In 2020, Tadashi Yanai, chairman of Fast Retailing, which operates the clothing company Uniqlo, donated 10 billion yen (approximately 1.123 billion won at that time) to Kyoto University for cancer immunity, stem cell, and coronavirus research. In 2018, Sumitomo Pharma completed the world's first facilities for producing iPS cell therapies. Dr. Masayo from RIKEN established a system using AI robots to produce iPS cell-based retinal cells that can treat over 800 patients within four months.

Japan's stem cell research hub. /Chosunilbo

Japan not only provided investment but also established supportive systems. In 2013, Japan introduced a system that allows conditional use of stem cell and gene therapies in advance if their safety is confirmed and expected to be effective.

Medicines and medical devices must demonstrate efficacy and safety through clinical trials and receive official approval before they can be commercialized. However, for diseases lacking adequate treatment alternatives, this system allows for rapid patient treatment without having to undergo all clinical trial processes. Public insurance coverage has also been extended to regenerative medicine therapies, including gene and stem cell therapies, paving the way for R&D to translate into sales.

Nature remarked, "Overall, Japan is leading the way into the commercialization era of stem cell therapies and in the coming years, iPS-based treatments could become a transformative point in the lives of actual patients."

☞Stem cell

A type of primitive cell that can differentiate into various human cells. The term "adult stem cell" refers to those derived from adult bone marrow or adipose tissue, while "embryonic stem cells" are obtained from fertilized eggs remaining after infertility treatment, and "cloned embryonic stem cells" are derived from a cloned embryo created by fusing a fully developed cell and an egg. Induced pluripotent stem cells (iPS cells) are made by inserting specific genes into fully developed cells to create them in the embryonic stem cell state.

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NEJM(2017) DOI: https://www.nejm.org/doi/10.1056/NEJMoa1608368

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※ This article has been translated by AI. Share your feedback **here.**

Feb. 13, 2026 Products

Print（PDF/138KB）

Regarding the Scheduled Review in Japan of the Allogeneic iPS Cell-Derived dopaminergic neural progenitor cells (AMCHEPRY)

Sumitomo Pharma Co., Ltd. (Head Office: Osaka, Japan; Representative Director, President and CEO: Toru Kimura) and RACTHERA Inc. (Head Office: Chuo-ku, Tokyo) submitted, on August 5, 2025, an application of manufacturing and marketing authorization for allogeneic iPS cell-derived dopaminergic neural progenitor cells (INN: raguneprocel) for indication of the improvement of motor functions during the off-time period of patients with advanced Parkinson's disease. Sumitomo Pharma and RACTHERA announced today that the regenerative medical product AMCHEPRY® has been listed as an item for deliberation on the agenda of the upcoming meeting of the Ministry of Health, Labour and Welfare's Pharmaceutical Affairs and Food Sanitation Council, Committee on Regenerative Medicine Products and Biotechnologies, scheduled to be held on February 19, 2026.

Following deliberation at the above committee, a decision will be made regarding the approval or disapproval of AMCHEPRY®, and other related matters. Any matters requiring disclosure will be communicated promptly. This development is expected to have no impact on the consolidated financial results for the fiscal year ending March 2026.

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Annex H – Institutional contact information (PROTON CRYOTECH)

For technical inquiries, equipment demonstrations, or collaborative initiatives, interested parties can contact:

PROTON CRYOTECH
Biomedical division of PROTON Europe
Exclusive worldwide distributor (excluding Asia) of all technologies manufactured by Ryoho Freeze Systems (Japan).
Address: Lluis Companys 117. 08393 Caldes d´Estrac. Barcelona. Spain

- **Showroom:** University of Barcelona. Torribera Food Campus.
- **Website: www.protoneurope.com** – (Information on PROTON technology in the biomedical and food sectors).
- **Email:** manuel@proton-europe.com
  Telephone: +34 627 925 103 (Manuel Ayllón, CEO).

Likewise, PROTON EUROPE can facilitate contact with associated Japanese institutions (Kyoto CiRA, NIBIOHN, etc.) for academic exchanges or technical visits if of interest.
Readers, especially physicians and researchers, are invited to join the international community exploring and applying this innovative magnetic freezing technology to usher biomedicine into a new era of possibilities.