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Safe and efficient method for cryopreservation of human induced pluripotent stem cell-derived neural stem and progenitor cells by a programmed freezer with a magnetic field

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ABSTRACT

Stem cells represent a potential cellular resource in the development of regenerative medicine approaches to the treatment of pathologies in which specific cells are degenerated or damaged by genetic abnormality, disease, or injury. Securing sufficient supplies of cells suited to the demands of cell transplantation, however, remains challenging, and the establishment of safe and efficient cell banking procedures is an important goal. Cryopreservation allows the storage of stem cells for prolonged time periods while maintaining them in adequate condition for use in clinical settings. Conventional cryopreservation systems include slow-freezing and vitrification both have advantages and disadvantages in terms of cell viability and/or scalability. In the present study, we developed an advanced slow-freezing technique using a programmed freezer with a magnetic field called Cells Alive System (CAS) and examined its effectiveness on human induced pluripotent stem cell-derived neural stem/progenitor cells (hiPSC-NS/PCs). This system significantly increased cell viability after thawing and had less impact on cellular proliferation and differentiation. We further found that frozen-thawed hiPSC-NS/PCs were comparable with non-frozen ones at the transcriptome level. Given these findings, we suggest that the CAS is useful for hiPSC-NS/PCs banking for clinical uses involving neural disorders and may open new avenues for future regenerative medicine.

1. Introduction

A growing body of evidence indicates that stem cells may serve as a useful source for regenerative medicine applications targeting certain refractory diseases. Neural stem and progenitor cells (NS/PCs) derived from human fetus and/or embryonic stem cells (ESCs) represent one potential source of transplantation approach for neurodegenerative diseases and neurotraumatic injuries, such as brain infarction and spinal cord injury (SCI). While NS/PCs can be incorporated into host tissues and restore nervous system functionality (Nakamura and Okano, 2013; Okano et al., 2013), the harvesting of such cells from a human fetus confronts legal and ethical challenges in many countries including Japan. Since the development of a new technique for establishing human induced pluripotent stem cells (hiPSCs) from somatic tissues (Takahashi et al., 2007), hiPSCs have become an attractive alternative cell source due to their ease of generation and comparative lack of ethical baggage.

In Japan, there is an ongoing project to establish a hiPSCs bank to provide cells for use in allogeneic transplantation (Nakatsuji et al., 2008; Okano and Yamanaka, 2014; Okita et al., 2011). We have previously reported the effectiveness of transplanting hiPSC-derived NS/PCs (hiPSC-NS/PCs) for SCI in rodents and non-human primates (Imaizumi et al., 2012; Kobayashi et al., 2012; Nori et al., 2011). Because it takes about six months to establish hiPSC-NS/PCs derived from an SCI patient's autologous somatic cells (Okada et al., 2008), at present it is impossible to perform auto-graft of iPSC-NS/PCs within the optimal therapeutic time window for sub-acute SCI (from 2 to 4 weeks after injury) (Nishimura et al., 2014; Okano

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and Yamanaka, 2014). To extend our results into potential clinical applications, therapeutic strategies will heavily rely on allogeneic transplantation of hiPSC-NS/PCs. We note here that hiPSC-NS/PCs may need to be stored properly to achieve favorable outcomes after transplantation. Cryopreservation makes it possible to store cells for prolonged time periods adequate for the clinical setting, although hiPSC-NS/PCs should be cryopreserved with high survival rates and minimal impact on cellular properties such as proliferation and developmental potency after freezing and thawing. Cryopreserved cells intended for clinical use must further be validated at given time points to avoid significant adverse outcomes such as failure to engraft into recipient tissue. However, few studies to date have evaluated NS/PC properties before and after freezing and thawing (Hancock et al., 2000; Ma et al., 2010; Milosevic et al., 2005; Paynter, 2008; Tan et al., 2007).

Currently preferred procedures for preparing cryopreserved cells are classified into two general types: slow-freezing and vitrification. The slow-freezing procedure has the beneficial aspect of enabling the preparation of large quantities of vials at one time, while the vitrification method is superior in terms of cell survival. Recently, the Cells Alive System (CAS) (ABI corporation Ltd., Abiko, Japan) was developed as a novel freezing technology and it has been used in the cryopreservation of various kinds of cells, including murine osteoblasts, rat bone tissue fragments, rat mesenchymal stem cells (MSCs), human periodontal ligament cells, and human ES cells (Abedini et al., 2011; Kaku et al., 2010, 2014, 2015; Kojima et al., 2013, 2015; Koseki et al., 2013; Lin et al., 2013) and proven to be a more effective method for cell preservation. However, it remains unclear whether this procedure is applicable to the banking of hiPSC-NS/PCs as well.

In this study, we used CAS to cryopreserve hiPSC-NS/PCs and examined the effects of cryopreservation process on hiPSC-NS/PCs. We determined a simple, optimized method designed for clinical use of these cells with improved cell viability without detrimental impact on cell properties using this system.

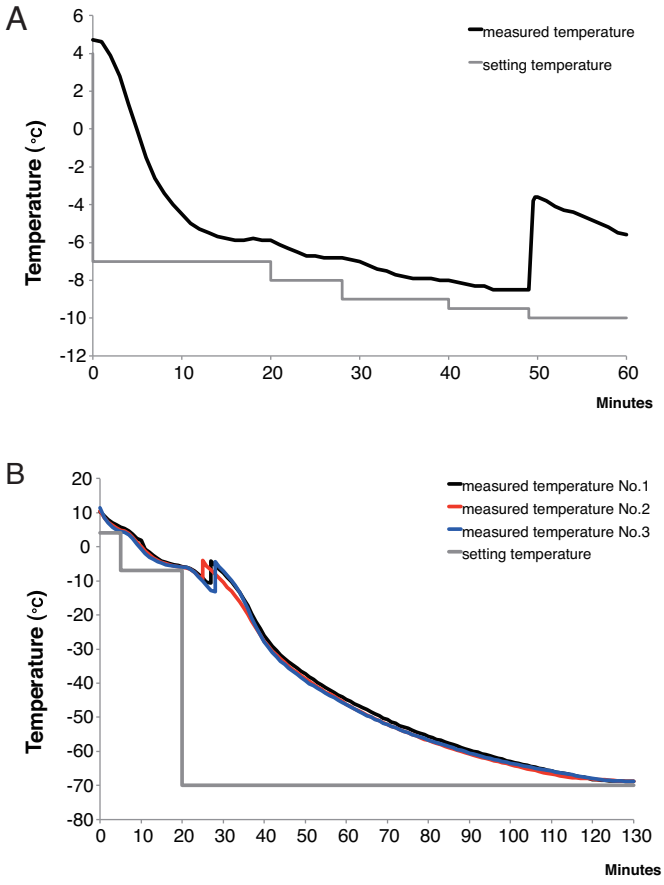

Fig. 1. Temperature of the cryopreservation solution. (A) Freezing temperature of cryopreservation solution (STEM-CELLBANKER). Lowering the setting temperature gradually, exothermic reaction was observed when actual temperature became about −8.5 °C. It indicates that freezing starts at this temperature. (B) Setting of the CAS programmed freezer and changes of the actual temperature. After the confirmation of the freezing temperature, we set the temperature of the CAS programmed freezer as shown in gray line. It is hold for 5 min at 4 °C, for 15 min at −7 °C (supercooling state) and plunged to −70 °C. In this setting, it took about 130 min to became −70 °C.

2. Materials and methods

2.1. Cell culture

The hiPSCs (clone 201B7) were cultured and maintained as described previously (Kobayashi et al., 2012; Nori et al., 2011; Takahashi et al., 2007). To perform neural induction of hiPSCs, we generated embryoid bodies (EBs) as described previously (Kobayashi et al., 2012; Nori et al., 2011). EBs were then enzymatically dissociated into single cells and cultured in suspension in serum-free media hormone mix (MHM) media for 10–14 days to allow the formation of neurospheres (Kobayashi et al., 2012). Neurospheres were dissociated into single cells and then cultured in the same manner for passage. The diameters of neurospheres were measured using inverted microscope (IX73, OLYMPUS Corporation, Tokyo, Japan). To evaluate cell viability, cells were enzymatically dissociated immediately after thawing in a water bath at 37 °C for 2 min and viable cells were counted by trypan blue exclusion test. Five visual fields chosen at random with a 4× objective lens were used to identify the diameter of hiPSC-NS/PCs spheres.

2.2. Cell freezing

After partially dissociating the hiPSC-NS/PCs, we counted the viable cells using the trypan blue exclusion test at three or six days after the last passage. A total of 2 or 5×10^6 cells were placed as neurospheres in a cryo-tube (Nunc CryoTubes, Thermo Fisher Scientific Inc., MA, USA) with 1 ml cryopreservation solution (STEMCELL-BANKER, ZENOAQ, Fukushima, Japan). For cryopreservation, the vials were placed in the CAS programmed freezer containing a magnetic field or a freezing container (Nalgene Mr. Frosty, Thermo Fisher Scientific, MA, USA). The alternating magnetic field with an induced electric field generated by the CAS freezer causes cells and water molecules to vibrate by a non-thermal mechanism and prevents the formation of intracellular ice crystals. As a result, improvement of cell viability would be achieved (Kaku et al., 2010, 2012, 2015). The electric current and frequency were adjusted to change the intensity of the magnetic field in the CAS. The freezing protocol was as follows: 4 °C for 5 min, −7 °C for 15 min, plunged to −70 °C (shown as the gray line in Fig. 1B); the electric current of CAS was adjusted as follows: 0.5, 0.4 and 0.3 A. We set the frequency of CAS to 30 Hz. To measure the actual temperature, thermocouple thermometer (testo 735-2, Testo, Kanagawa, Japan) with a sheath-type flexible probe was used in every minute and it was repeated at least three times.

2.3. Immunocytochemistry

To perform immunocytochemistry in spheres, we centrifuged the spheres at 1000 rpm for 5 min. Cells were collected using iPGell (GenoStaff, Tokyo, Japan) and postfixed for 1 h in 4% paraformaldehyde (PFA), soaked 4 h in 10% sucrose, 4 h in 20% sucrose, and then overnight in 30% sucrose, following the manufacturer's instructions. We then embedded the spheres in Optimal Cutting

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