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Highly efficient cryopreservation of human induced pluripotent stem cells using a dimethyl sulfoxide-free solution

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ABSTRACT Human induced pluripotent stem (hiPS) cells have great potential for regenerative medicine and drug discovery. It is essential to establish highly efficient and reliable methods for hiPS cell cryopreservation. We examined cryopreservation of hiPS cells by the vitrification method using a dimethyl sulfoxide Me_2SO-free and serum-free medium, VS2E, that uses Euro-Collins solution as a base with 40% (v/v) ethylene glycol and 10% (w/v) polyethylene glycol as cryoprotectants. This combination of vitrification and cryoprotectants resulted in a higher recovery rate of hiPS cells than with a commercially-available vitrification solution, DAP213, which contained Me_2SO and serum components. After vitrification and warming, hiPS cells were cultured easily. Even after several subculturing steps, cells expressed undifferentiated cell markers, such as Oct-3/4 and SSEA-4, and also exhibited alkaline phosphatase activity. The pluripotency of hiPS cells was maintained, as demonstrated by teratoma formation upon hiPS cell transplantation into severe combined immunodeficient mice. Thus, we successfully preserved hiPS cells under liquid nitrogen with high efficiency using Me_2SO-free vitrification solution and rapid cooling.

KEY WORDS: *cryopreservation, human induced pluripotent stem (iPS) cell, vitrification, Me_2SO-free, ethylene glycol*

Introduction

Human embryonic stem (hES) and induced pluripotent stem (hiPS) cells are important in regenerative medicine and in drug discovery due to their pluripotent potential (Keller and Snodgrass, 1999; Ameen *et al*., 2008; Nakagawa *et al*., 2008; Takahashi *et al*., 2007; Yu *et al*., 2007). It is essential to establish methods for stably storing these stem cells, especially hiPS cells derived from patients with obstinate diseases. Cryopreservation using conventional slow-freezing methods results in low hiPS cell survival after thawing; further, a long culture period (at least 2 weeks) is required after thawing before experiments can be conducted (Reubinoff *et al*., 2001; Fujioka *et al*., 2004). Efficient cryopreservation and subsequent culture thus remain an obstacle to the effective use of hiPS.

Cryopreservation protocols developed for hES cells (Reubinoff *et al*., 2001; Fujioka *et al*., 2004; Richards *et al*., 2004; Ji *et al*., 2004) have also been used for hiPS cell cryopreservation. Although cell survival is improved compared to conventional methods, there is still room for improvement. Most cryopreservation media contain fetal bovine serum or serum-replacement and dimethyl sulfoxide (Me_2SO). Ideally, components from animal serum or serum-replacement should not be included in the culture media to reduce the risks of infection by unknown pathogens in future regenerative medicine applications. Me_2SO [~1% (v/v)] has been used for ES cell differentiation into mesendoderm (Ameen *et al*., 2008), and cryopreservation with slow freezing method using Me_2SO reportedly induces ES cell differentiation (Katkov *et al*., 2006). Therefore, it is desirable to develop a cryopreservation medium that lacks both Me_2SO and animal-derived components. Recently, the cryopreservation recovery ratios of hES and hiPS cells was improved by using Rho-associated coiled-coil kinase (ROCK) inhibitor (Martin-Ibañez *et al*., 2008; Li *et al*., 2009; Claassen *et al*., 2009; Mollamohammadi *et al*., 2009); however, cryopreservation with the ROCK inhibitor is

Abbreviations used in this paper: DSC, differential scanning calorimeter; hES, human embryonic stem cell; hiPS, human induced pluripotent stem cell; SPR, surface plasmon resonance; SUV, small unilamellar vesicle; tBLM, tethered bilayer membrane.

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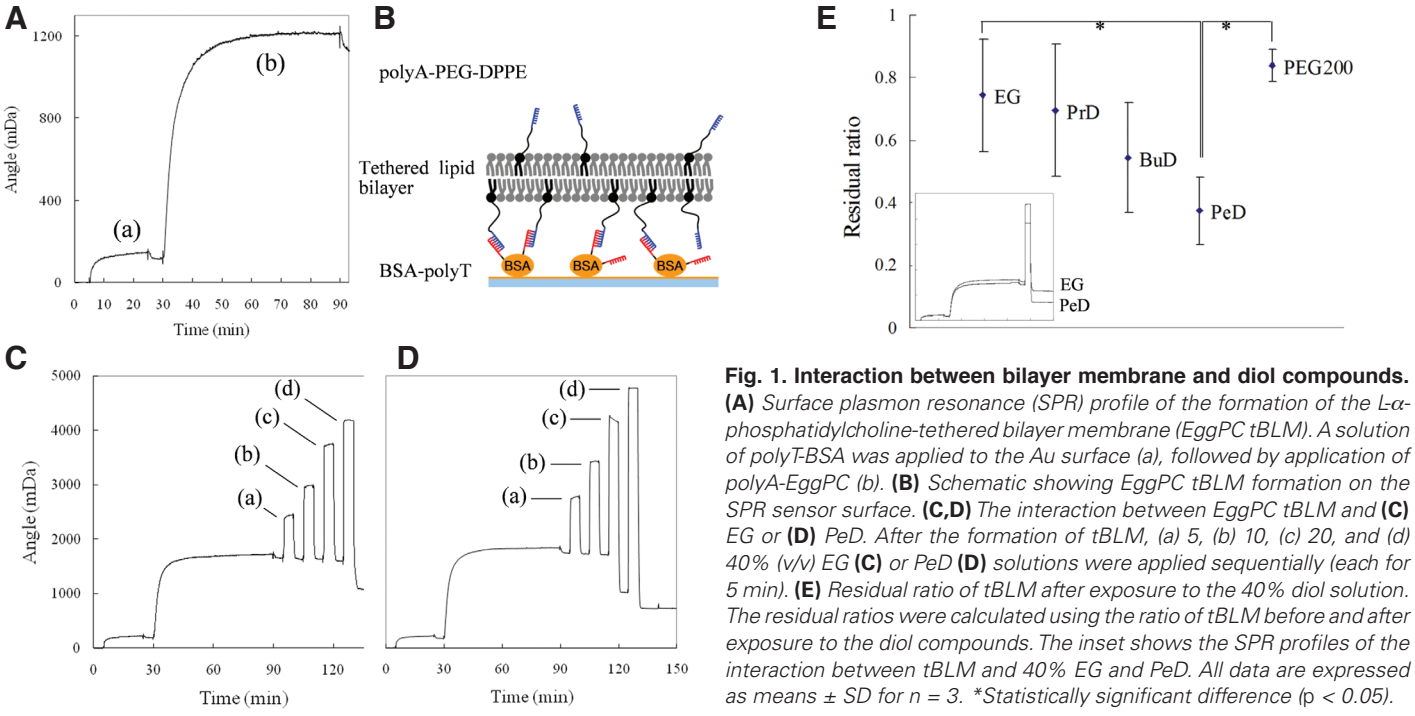

Fig. 1. Interaction between bilayer membrane and diol compounds. **(A)** *Surface plasmon resonance (SPR) profile of the formation of the L-α-phosphatidylcholine-tethered bilayer membrane (EggPC tBLM). A solution of polyT-BSA was applied to the Au surface (a), followed by application of polyA-EggPC (b).* **(B)** *Schematic showing EggPC tBLM formation on the SPR sensor surface.* **(C,D)** *The interaction between EggPC tBLM and* **(C)** *EG or* **(D)** *PeD. After the formation of tBLM, (a) 5, (b) 10, (c) 20, and (d) 40% (v/v) EG* **(C)** *or PeD* **(D)** *solutions were applied sequentially (each for 5 min).* **(E)** *Residual ratio of tBLM after exposure to the 40% diol solution. The residual ratios were calculated using the ratio of tBLM before and after exposure to the diol compounds. The inset shows the SPR profiles of the interaction between tBLM and 40% EG and PeD. All data are expressed as means ± SD for n = 3. *Statistically significant difference (*$p < 0.05$*).*

very time consuming, and the inhibitor might affect cell function. Thus, a simpler and more reliable method is needed.

Our group recently developed vitrification solutions for the cryopreservation of pancreatic islets and primate ES cells (Agudelo and Iwata, 2008; Agudelo *et al.*, 2009; Nishigaki *et al.*, 2010). These solutions do not contain Me_2SO and animal-derived components; rather, the main components are ethylene glycol (EG) and Euro-Collins solution. Our results suggested that diol compounds could replace Me_2SO for cryopreservation. In the present study, we systematically examined the use of diol compounds, including EG, propane diol (PrD), butane diol (BuD), pentane diol (PeD), and polyethylene glycol (MW 200; PEG200) to improve the survival rate of hiPS cells after cryopreservation by vitrification. An alkaline phosphatase activity assay and immunostaining for Oct4 and SSEA-4 were performed to evaluate the differentiation status of the hiPS cells. The potency of hiPS cells to differentiate into three germ lines was also examined by transplantation of hiPS cells into severe combined immunodeficient (SCID) mice after the vitrification and warming procedure.

Results and Discussion

Effects of diol solutions on tBLM

In this study, we examined the use of four diol compounds, HO-$(CH_2)_n$-OH (n = 2-5), and poly(ethylene glycol) HO-$(CH_2CH_2O)_n$-H as cryoprotectants in a vitrification solution for hiPS cells. Surface plasmon resonance (SPR) was employed to investigate the interaction between a cell membrane and the diol compounds *in vitro*. A tethered bilayer membrane (tBLM) was formed on the SPR sensor surface and exposed to solutions with different concentrations of diols. tBLMs were prepared using the vesicle fusion method on a hydrophilic surface (Taylor *et al.*, 2007; Chung *et al.*, 2009). First, polyT-BSA was adsorbed on the SPR sensor surface, and then a suspension of polyA-PEG-DPPE-modified SUVs was applied to the surface. PolyT-BSA was effectively adsorbed on the Au surface (Fig. 1Aa). The largest shift increase (~1 DA) was observed in the SPR profile when polyA-EggPC was applied to the polyT-BSA-modified sensor surface (Fig. 1Ab), indicating the formation of tBLM as shown schematically in Fig. 1B.

A cell membrane consists of a thin lipid bilayer that contains membrane proteins. The effects of diol compounds on cell membranes were examined by SPR using tBLM as a cell membrane model. The EggPC tBLM was exposed to increasingly concentrated diol solutions containing 5, 10, 20, and 40% (v/v) of diol in PBS for 5 min each, and then the surfaces were washed with PBS for 5 min. The SPR signal intensities were monitored to determine whether diol compounds damaged the tBLM. Fig. 1 C,D show the SPR profiles

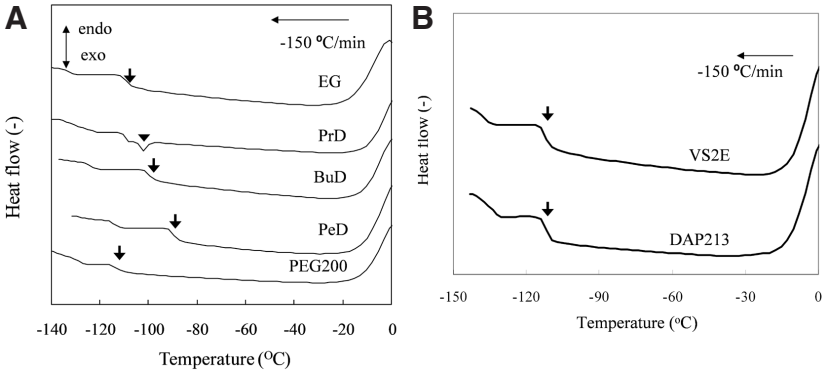

Fig. 2. Thermal analysis of (A) 40% diol compound solutions in Euro-Collins solution and (B) VS2E and DAP213 solution using differential scanning calorimetry (DSC). *The chamber was cooled from 0 to -140°C at a rate of -150°C/min. Arrows indicate changes in the slope, and an arrowhead indicates an exothermic peak.*

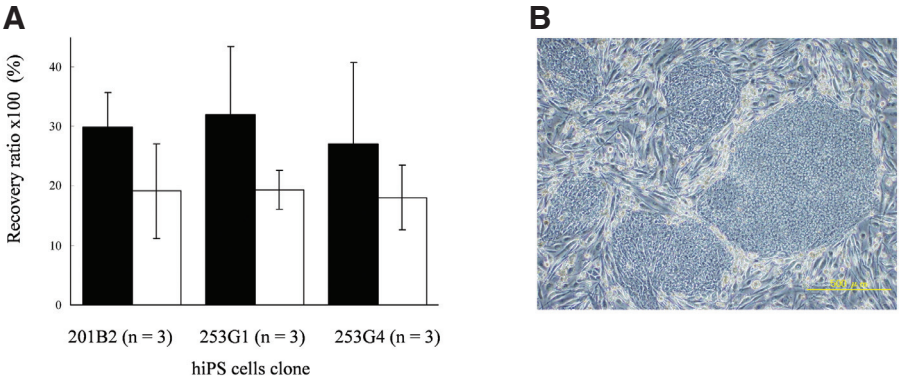

Fig. 3. Recovery rates of hiPS cells and colonies formed by hiPS cells after freezing and thawing. (A) *Recovery rates of hiPS cells (clones: 201B2, 253G1, 253G4) 1 day after the cryopreservation and thawing procedure. hiPS cell were cryopreserved in VS2E (black bars) or DAP213 (white bars). The recovery rates were calculated by comparing the number of colonies 1 day after thawing/culture to the number of colonies formed by cells that were not cryopreserved. Results are expressed as means ± SD for n = 3.* **(B)** *A phase contrast microscope image of cultured 253G1 hiPS cells that were cryopreserved in VS2E 3 days after thawing. Scale bar: 500 μm.*

when tBLMs were exposed to diol solutions of 5, 10, 20, and 40% (v/v) EG or PeD, respectively. There was no change in the SPR signal intensity when the tBLM was washed with PBS after a 5, 10, or 20% EG solution was applied, but the SPR signal intensity decreased after the 40% EG was applied. These results suggested that the tBLM remained intact when exposed to up to 20% EG but was damaged upon exposure to 40% EG. For PeD, the decrease in the SPR signal intensity began upon exposure to 20% PeD; thus, PeD interacted with or damaged the lipid bilayer membrane at lower concentrations than did EG. These experimental results indicated that the greater the hydrophobicity of the diol solution, the greater the damage to the cell membrane.

Fig. 1E shows the relative amounts of the residual lipid bilayer membrane when tBLM was exposed to 40% diol solutions. The amount of residual membrane decreased with increased diol molecular weight. The exception was PEG200: although it was the highest molecular weight compound used in this study, it did not damage the tBLM very much. PEG is a hydrophilic molecule, so presumably there was little or no interaction with the lipid bilayer membrane. In general, hydrophobic diol compounds with longer alkyl chain damaged the bilayer membrane.

Thermal properties of cryopreservation solutions during the cooling process

Effective vitrification is important for cryopreservation of hiPS cells. DSC analyses were carried out to monitor ice crystal formation in solutions of diol compounds in Euro-Collins solution. Fig. 2A shows DSC charts of 40% (v/v) diol solutions during cooling at a rate of -150°C/min. The slopes of the DSC curves of EG, BuD, PeD, and PEG200 solutions changed at around -110, -100, -90, and -115°C (indicated by arrows). Those points indicate when the glass-transition phase change occurred. When the solutions in cryotubes were immersed directly into liquid nitrogen, the solutions remained transparent. These data indicate that the diol solutions were vitrified effectively. However, an exothermic peak was observed for the PrD solution at around -105°C, as indicated by an arrowhead in Fig. 2A. This could be attributed to crystallization of the PrD solution, and indeed it has been reported that PrD tends to form intermolecular hydrogen bonds in water (Takamuku *et al.*, 2008), indicating long-range ordering. This might lead to crystallization.

The thermal analyses suggested that EG, BuD, PeD, and PEG200 were suitable for use in a vitrification solution. The SPR studies (Fig. 1) indicated that diols with shorter alkyl chain were less harmful to the artificial cell membrane.

Vitrification of hiPS cells

A vitrification solution of human ES cells (VS2E) was prepared using EG, PEG200, and Euro-Collins solution (Nishigaki *et al.*, 2010). We examined various diols and their different concentrations to find a more suitable diol than EG for vitrification of hiPS cells (*see Supplemental information*). Although we had expected to find a more suitable diol than EG for vitrification, the results shown in *Supplemental Fig. 1 and 2* indicated that EG was the most suitable diol for use in a vitrification solution. In this study, we examined VS2E solution to cryopreserve hiPS cells, because various characters of hiPS cells are similar to those of hES cells.

A commercially available solution that contains Me_2SO and serum components, DAP213, is currently used for cryopreservation of ES cells and iPS cells. We compared vitrification using our solution, VS2E, with vitrification using DAP213. The compositions of these solutions are shown in Table 1. These cryopreservation solutions were analyzed using DSC (Fig. 2B) at a cooling rate of -150°C/min. No exothermic peak was observed for crystallization in either the DAP213 or VS2E solution. The slope changes around -110°C, indicating the glass-transition phase change (arrows in Fig. 2B), were observed for both solutions. Both solutions remained transparent after immersion into liquid nitrogen. These results indicate the two solutions were vitrified by rapid cooling.

hiPS cells (253G1, 253G4, and 201B2) were preserved in liquid nitrogen using a combination of rapid cooling and vitrification solution VS2E or DAP213. The recovery rates of these hiPS after vitrification and warming are summarized in Fig. 3A, and a phase contrast microscope image of hiPS cells cultured for 3 days is shown in Fig. 3B. The recovery rates of hiPS cell clones 201B2, 253G1, and 253G4, preserved by vitrification using VS2E were 29.8 ± 5.8% (n = 3), 32.0 ± 11.4% (n = 3), and 27.1 ± 13.6% (n = 3), respectively. There was no significant difference in the

TABLE 1

COMPOSITION OF THE CRYOPRESERVATION SOLUTIONS

	VS2E	DAP213
Me_2SO % (v/v)	–	14.2
EG % (v/v)	40	–
PG % (v/v)	–	22
PEG % (w/v)	10	–
Acetamide % (w/v)	–	5.9
DMEM/F12 with KSR	–	+
Euro-Collins	+	–

Me_2SO: dimethyl sulfoxide; EG: ethylene glycol; PG: propylene glycol; PEG: polyethylene glycol (MW1000); DMEM/F12: Dulbecco's modified Eagle medium/F12; KSR: knockout serum replacement; Euro-Collins: 34.95 g/L dextrose, 7.3 g/L K_2HPO_4, 2.04 g/L KH_2PO_4, 1.12 g/L KCl, and 0.84 g/L $NaHCO_3$.

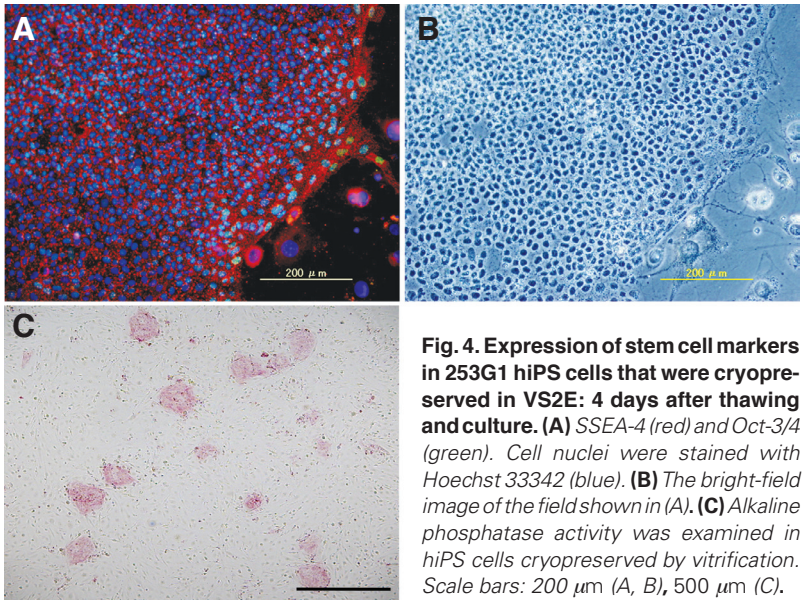

Fig. 4. Expression of stem cell markers in 253G1 hiPS cells that were cryopreserved in VS2E: 4 days after thawing and culture. (A) *SSEA-4 (red) and Oct-3/4 (green). Cell nuclei were stained with Hoechst 33342 (blue).* **(B)** *The bright-field image of the field shown in (A).* **(C)** *Alkaline phosphatase activity was examined in hiPS cells cryopreserved by vitrification. Scale bars: 200 μm (A, B),* 500 *μm (C).*

recovery rates of the different clones. In addition, all of the hiPS cells proliferated well and those could be subcultured 3 days after seeded. These results indicated that the VS2E solution developed for vitrification of hES cells was suitable for cryopreservation of hiPS cells as well. In contrast, the recovery rates of hiPS cell clones 201B2, 253G1, and 253G4, preserved by vitrification using the commercially available solution, DAP213, were 19.3 ± 3.3% (n = 3), 18.0 ± 5.4% (n = 3), and 19.1 ± 8.0% (n = 3), respectively.

Although the differences in recovery rates using VS2E and DAP213 were not significant, the recovery rates for the VS2E solution tended to be higher than those for DAP213. Although DAP213 is an effective vitrification solution (Fig. 3A), VS2E is preferable because it is free of Me_2SO and serum components. In a series of experiments, we also cryopreserved hiPS cells (clone 253G1) using a conventional slow freezing method with 10% Me_2SO. Only a few colonies were formed after cryopreservation and thawing (data not shown) and it took 3 to 4 weeks to subculture hiPS cells. These results indicated that the slow freezing method is not suitable for cryopreservation of hiPS cells.

After hiPS cells were cryopreserved with VS2E and subsequently cultured for 4 days, colonies were analyzed immunohistochemically for undifferentiated cell markers SSEA-4 and Oct3/4 (Thomson *et al.*, 1998; Sperger *et al.*, 2003). The alkaline phosphatase activity of the hiPS cells was visualized after fixation with PFA. The hiPS cells exhibited alkaline phosphatase activity and expressed SSEA-4 and Oct3/4 (Fig. 4). The undifferentiated state was thus maintained in the hiPS cells after cryopreservation in VS2E under liquid nitrogen, thawing, and culture.

The pluripotency of the hiPS cells was examined by transplantation into SCID mice. Twelve weeks after transplantation, the teratomas formed from the injected cells were removed for histological examination. Fig. 5 shows HE-stained tissue sections from the teratomas. Endodermal epithelium, mesoderm-derived cells (muscle, cartilage), and neuroepithelium were identified in teratomas formed from 253G1 hiPS cells. Thus, histological analyses of the teratomas revealed that cryopreserved hiPS cells vitrified using VS2E and preserved under liquid nitrogen retained their pluripotency.

We meet some difficulties in cryopreservation of cells by vitrification. Those are troublesome procedures, a tube container with small diameter, the use of Me_2SO, and contamination risk from the direct contact with liquid nitrogen. In this study, we made some improvement in cell vitrification, such as use of a conventional cryotube, the simple solution without Me_2SO, and simple procedure.

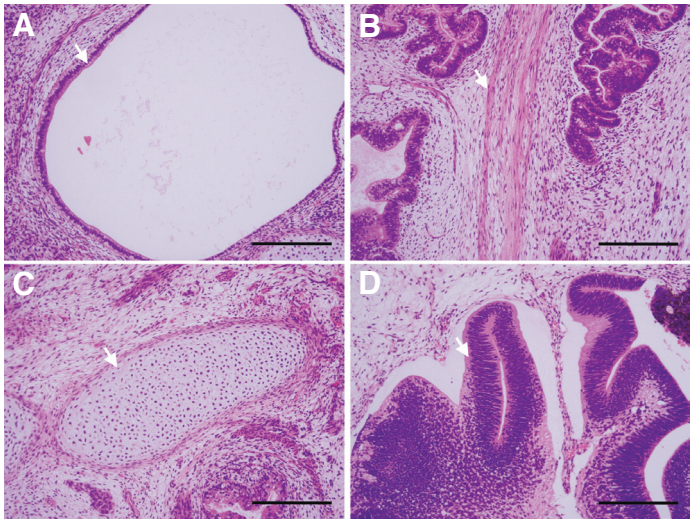

Fig. 5. Teratoma formation in SCID mice after transplantation of 253G1 hiPS cells cryopreserved in VS2E solution. *Arrows indicate* **(A)** *endodermal epithelium,* **(B)** *muscle,* **(C)** *cartilage, and* **(D)** *neuroepithelium. Scale bars: 200 μm.*

Conclusion

We successfully cryopreserved and recovered hiPS cells using a vitrification solution composed of 40% (v/v) EG and 10% (v/v) PEG in Euro-Collins solution. EG was the most suitable diol compound for use in the vitrification solution. hiPS cells could be preserved under liquid nitrogen using VS2E solution combined with rapid cooling. The recovery rates were around 30%, and the hiPS cells maintained pluripotency after cryopreservation using liquid nitrogen and subsequent thawing and culture. The cryopreservation solution VS2E thus has great potential for use in experimental research as well as in medical applications of hiPS cells.

Experimental Protocols

Surface plasmon resonance (SPR)

In this paper, we examined the interaction between diol compounds and a tethered bilayer membrane (tBLM) *in vitro* using an SPR apparatus to elucidate the influence of diol compounds on a living cell membrane (Pavey *et al.*, 1999; Green *et al.*, 1997). The tBLM was fabricated by the vesicle fusion method using small unilamellar vesicles (SUVs) on a hydrophilic surface (Taylor *et al.*, 2007; Chung *et al.*, 2009).

Synthesis of polyA-PEG-DPPE and polyT-BSA

α-*N*-Hydroxysuccinimidyl-ω-maleimidyl poly(ethylene glycol) (NHS-PEG-Mal, Mw: 5000) and 1,2-distearyl-*sn*-glycerol-3-phosphatidylethanolamine (DSPE) were purchased from the NOF Corporation (Tokyo, Japan). Mal-PEG-DPPE was synthesized by first dissolving NHS-PEG-Mal (180 mg), triethylamine (50 μL, Nacalai Tesuque, Kyoto, Japan), and DPPE

(20 mg) in dichloromethane (Nacalai Tesuque) and stirring for 36 h at RT (Teramura *et al.*, 2010a; Teramura *et al.*, 2010b; Teramura *et al.*, 2007). A white powder (190 mg, yield 80%) was obtained by precipitation of the reaction mixture with diethyl ether. ^{1}H-NMR ($CDCl_3$, 400 MHz, δ ppm): 0.88 (t, 6H, -CH_3), 1.25 (br, 56H, -CH_2-) 3.64 (br, 480H, PEG), 6.71 (s, 2H, -HC=CH-, maleimide).

Poly(adenosine phosphate) (20) (polyA) and poly(thymidine phosphate) (20) (polyT) carrying protected SH group at the 5'-ends were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). PolyA with a 5'-end SH group was prepared by reduction of the disulfide bond with DTT according to the manufacturer's instructions. The SH groups were conjugated to the Mal-PEG-lipid to prepare polyA-PEG-lipid. PolyA-SH (1.0 mg) was mixed with Mal-PEG-DPPE (5.0 mg) in PBS and incubated for 24 h at RT to prepare polyA-PEG-lipid (500 μg/mL in PBS). PolyT-PEG-DPPE was prepared the same way as polyA-PEG-DPPE.

Bovine serum albumin (BSA) (Cohn V fraction, Sigma) in PBS (500 μL of a 20 mg/mL solution) was mixed with 20 μL of 62.2 mg/mL sulfo-EMCS (*N*-(6-maleimidocaproyloxy) sulfo-succinimide, sodium salt; Thermo Scientific, USA) and incubated for 2 h at RT to introduce maleimide groups onto the BSA. Maleimide-conjugated BSA (BSA-Mal) was purified with a Sephadex™ G-25 M column (GE Healthcare UK Ltd., Buckinghamshire, UK). BSA-Mal solution (8.2 μL of a 10 mg/mL solution) was mixed with 90 μL of a 1.78 mg/mL polyT-SH solution, and the reaction mixture was incubated for 2 h at RT. This reaction mixture was used as a polyT-BSA solution.

Small unilamellar vesicles (SUVs)

L-α-phosphatidylcholine (EggPC, Sigma) was dissolved in chloroform and put in a flask. The chloroform was removed to prepare a dry thin lipid film using a rotary evaporator. PBS was added to the lipid film and stirred vigorously at 4°C for 4 days to prepare lipid vesicles (lipid concentration = 10 mg/mL). The suspension was then extruded through membrane filters with pore size 0.8 μm, 0.22 μm (2 times), and 0.1 μm (10 times) to form SUVs (~100 nm diameter). PolyA-EggPC was prepared by incubation of SUVs with polyA-PEG-DPPE for 1 h at 37°C.

SPR measurements

Glass plates (BK7, refractive index: 1.515, size: 25 × 25 × 1 mm, Arteglass Associates Co., Kyoto, Japan) were immersed for 5 min in a piranha solution (a 7:3 mixture of concentrated sulfuric acid and 30% hydrogen peroxide), washed three times with deionized water, rinsed three times with Milli-Q water and three times with 2-propanol, and finally stored in 2-propanol until use. The glass plates were dried under a stream of nitrogen gas and then mounted on the rotating stage of a thermal evaporation coating apparatus (V-KS200, Osaka Vacuum, Ltd., Osaka, Japan). A 1-nm chromium layer was deposited, and then a 49-nm gold layer was deposited.

This study used an SPR apparatus that was constructed in our laboratory (Hirata *et al.*, 2000). A flow chamber with a gold-covered glass plate was placed on the prism of the SPR apparatus, and PBS was circulated at a flow rate of 4.0 mL/min in the flow chamber for at least 5 min. The reflectivity of a *p*-polarized HeNe laser beam (λ = 632.8 nm) from the sample unit was monitored as a function of the incident angle. The least-squares method of the quadratic function was applied to points around the point of maximum loss of reflectivity, and the minimum point described by this fitted curve was defined as the resonance angle. The incident angle was then fixed at 0.5° less than the resonance angle. For construction of a tethered bilayer membrane (tBLM), a 30 μg/mL polyT-BSA solution in PBS was introduced into the flow chamber, allowed to adsorb onto the Au surface for 20 min, and then a 100 μg/mL polyA-EggPC solution in PBS was circulated for 1 h. To determine whether the diol solutions damaged the membrane, the EggPC tBLM was exposed to diol solutions [5, 10, 20, and 40% (v/v) EG or PeD in PBS] for 5 min, then washed with PBS for 5 min. To express the degree of damage to the EggPC tBLM, the residual ratio of the EggPC tBLM was calculated as follows:

Residual ratio = (SPR angle shift after exposure to the diol solution) / (SPR angle shift of the EggPC tBLM from the sensor surface).

DSC measurements

A differential scanning calorimeter (DSC, Diamond DCS, PerkinElmer, Inc. MA, USA) was used for thermal analysis of the cryopreservation solutions during the cooling process. The sample solutions were cooled from 0°C to -140°C at a rate of -150°C/min.

hiPS cell culture

hiPS cell line 253G4, established by transfection with OCT3/4, SOX2, and KLF4 (Nakagawa *et al.*, 2008), and cell line 201B2, established by transfection with OCT3/4, SOX2, KLF4, and MYC, were the kind gift of Prof. Shinya Yamanaka. The hiPS cell line 253G1, established by transfection with OCT3/4, SOX2, and KLF4, was obtained from the RIKEN cell bank, Japan. Undifferentiated hiPS cells were maintained on a feeder layer of SNL76/7 cells (Thomas *et al.*, 1987) treated with mitomycin C (Wako Pure Chemical, Osaka, Japan). SNL76/7 cells are a mouse fibroblast STO cell line that expresses the neomycin-resistance gene cassette and LIF (ECACC, UK). The undifferentiated hiPS cells and SNL76/7 feeder cells were cultured in Dulbecco's modified Eagle medium/F12 (DMEM/F12, Sigma) supplemented with 20% (v/v) knockout serum replacement (KSR; Invitrogen, Carlsbad, CA), 0.1 mM nonessential amino acid (NEAA, Invitrogen), 2 mM L-glutamine (Sigma), 0.1 mM 2-mercaptoethanol (Sigma), 5 mM sodium hydroxide, and 5 ng/mL FGF2 (Kaken Pharmaceutical Co., Ltd., Tokyo, Japan) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The hiPS cells were subcultured every 4–5 days using 0.25% (v/v) trypsin and 0.1 mg/mL collagenase (Type S-1, Nitta Gelatin Inc., Osaka, Japan) in PBS (-) supplemented with 20% (v/v) KSR and 1 mM calcium chloride. The hiPS cells were suspended in PBS as cell clumps, but not single cells. SNL76/7 cells were routinely maintained in DMEM (Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS, Equitech-Bio, Inc. TX, USA).

Vitrification of hiPS cells

Liquid nitrogen preservation

We examined the cryopreservation of hiPS cells using VS2E vitrification solution as well as the DAP213 vitrification solution developed by Fujioka *et al.*, 2004. The compositions of the solutions are listed in Table 1. The VS2E and DAP213 solutions were sterilized by filtration through a membrane (φ: 0.22 μm) before use. Confluent hiPS cells in 60 mm dishes were harvested by treatment with trypsin and collagenase as described above. A pellet of hiPS cells was collected by centrifugation at 1000 rpm for 5 min and resuspended in 200 μL of a vitrification solution in a cryotube (1.8 mL, CryoTube™ Vials, Nunc™, Denmark). The cryotube was immediately immersed in liquid nitrogen and stored for 7 days until recovery rate determination was conducted.

Recovery rate of hiPS cells after liquid nitrogen preservation

Culture medium (1 mL) pre-warmed to 37°C was added directly to vitrified hiPS cells (200 μL) in the cryotube, followed by rapid pipetting. The hiPS cell suspension was immediately transferred to a centrifuge tube and spun at 1000 rpm for 3 min at RT. The supernatant was removed, the cell pellet was resuspended in 4 mL of culture medium, and the cell suspension was applied to a feeder-layer of SNL76/7 in a culture dish and cultured at 37°C, 5% CO_2. In the procedure, we carefully treated cells not to disperse into single cells, but to maintain cell clumps, because hiPS cells hardly proliferate when they were seeded as a single cell.

Cell colonies were observed under a phase contrast microscope after 1 day of culture. For a control experiment, hiPS cells without cryopreservation were seeded onto a SNL feeder-layer. To determine the recovery rates of hiPS cells after cryopreservation, cell colonies formed on the SNL cell layer were counted 1 day after culture. Recovery rates were calculated as follows:

Recovery rate (%) = 100 ×(the number of cell colonies formed by hiPS cells after preservation under liquid nitrogen) / (the number of cell colonies formed by hiPS cells without preservation).

Pluripotency of hiPS cells after liquid nitrogen preservation

In vitro histochemical analysis of hiPS cells after preservation in liquid nitrogen

After thawing and 4 days of culture, cells were fixed in 4% (w/v) paraformaldehyde (PFA) in PBS for 15 min and permeabilized by treatment with 0.2% (v/v) Triton X-100 solution for 15 min at RT. After treatment with the Blocking One® reagent (Nacalai Tesque) at RT for 1 h, cells were incubated with primary antibodies for 12 h at 4°C. The dilution ratios of the primary antibodies were as follows: mouse anti-SSEA-4, 1:400 (Chemicon, CA), and rabbit anti-Oct3/4, 1:200 (Santa Cruz Biotechnology, CA) (diluted in Blocking One® solution). The sample cells were washed in 0.05% (w/v) polyoxyethylene sorbitan monolaurate (Tween 20, in PBS, Wako) three times and then incubated with secondary antibodies for 2 h at RT. The dilution ratios of the secondary antibodies were as follows: Alexa Fluor® 488-labeled goat anti-mouse IgG, 1:500 (Invitrogen), and Alexa Fluor® 594-labeled goat anti-rabbit IgG, 1:500 (Invitrogen) (diluted in Blocking One® solution). To stain cell nuclei, the sample cells were incubated with Hoechst 33342 fluorescent dye (Dojindo Laboratories, Kumamoto, Japan) at a dilution of 1:500 in PBS for 15 min at RT. After washing with PBS, the sample cells were observed under a fluorescent microscope (IX71, Olympus Optical Co. Ltd., Tokyo, Japan). The alkaline phosphatase activity of the hiPS cells was visualized after fixation with PFA using the Alkaline Phosphatase Substrate Kit III (Vector Laboratories, Burlingame, CA, USA).

Teratoma formation by hiPS cells after preservation in liquid nitrogen using VS2E

hiPS cells were preserved under liquid nitrogen using VS2E and cultured for 1 passage. Cells were treated with 10 μM Y-27632 (Wako) for 1 h at 37°C before collection from the 100-mm culture dishes. Cells from a single dish were subcutaneously injected into the backs (right and left sides) of SCID mice. After 12 weeks, teratomas were removed and fixed in 4% (w/v) PFA solution overnight. Paraffin-embedded tissue sections (4 μm) were prepared using standard methods and stained with hematoxylin and eosin (HE) for visual examination.

Statistical analysis

Comparisons between two groups were made using Student's *t*-tests, and $p < 0.05$ was considered statistically significant. All statistical calculations were performed using statistical software (JMP 6.0).

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