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 Me₂SO-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 Me₂SO 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 Me₂SO-free vitrification solution and rapid cooling.

KEY WORDS: cryopreservation, human induced pluripotent stem (iPS) cell, vitrification, Me₂SO-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 (Me₂SO). 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. Me₂SO [~1% (v/v)] has been used for ES cell differentiation into mesendoderm (Ameen et al., 2008), and cryopreservation with slow freezing method using Me₂SO reportedly induces ES cell differentiation (Katkov et al., 2006). Therefore, it is desirable to develop a cryopreservation medium that lacks both Me₂SO 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.
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$_2$SO 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$_2$SO 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$_2$CH$_2$O)$_n$-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...
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 b'ayer 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.

| TABLE 1 |
| COMPOSITION OF THE CRYOPRESERVATION SOLUTIONS |
|---|---|
| VS2E | DAP213 |
| M2SO % (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 | - | - |
| Acetamide | 5.0 | - |

M2SO: 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 KHPO4, 2.04 g/L KH2PO4, 1.12 g/L KCl, and 0.84 g/L NaHCO3.
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).

**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-Hydroxy succinimidyld-α-Maleimidyl poly(ethylene glycol) (NHS-PEG-Mal, Mw: 5000) and 1,2-distearoyl-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 Tesque) 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, 6 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 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 accor-
ding 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 \(\mu\)g/mL in PBS). PolyT-PEG-DPPE was
prepared in the same way as polyA-PEG-DPPE.
Bovine serum albumin (BSA) (Cohn V fraction, Sigma) in PBS (500
\(\mu\)L of a 20 mg/mL solution) was mixed with 20 \(\mu\)L of 62.2 mg/mL sulfo-
EMCS (N-(6-maleimidocaproyloxy)sulfo-succinimid, 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 \(\mu\)L of a 10 mg/mL solution) was mixed with 90 \(\mu\)L
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-\(\alpha\)-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 \(\mu\)m, 0.22 \(\mu\)m (2 times), and 0.1 \(\mu\)m (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 x 25 x 1 mm, Artglass
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
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 (\(\lambda = 632.8 \) 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 \(\mu\)g/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 \(\mu\)g/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 ProD 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 mg/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 subsampled 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 (\(\cdot\)) 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 vitrifica-
tion solution as well as the DAP213 vitrification solution developed by
Fujoka 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 (\(\phi = 0.22 \) 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 \(\mu\)L of a vitrification solution in a cryotube
(1.8 mL, CryoTube™ Vials, Nunc™, Denmark). The cryotube was imme-
diately 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 vit-
riified hiPS cells (200 \(\mu\)L) 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 \(\times\) (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% (w/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 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 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 VSE2

hiPS cells were preserved under liquid nitrogen using VSE2 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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References


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