Human Reproduction Vol.20, No.7 pp. 1779–1785, 2005 doi:10.1093/humrep/deh854
Advance Access publication March 10, 2005

Cryopreservation of human embryonic stem cells without the use of a programmable freezer

Sung Yun Ha[1], Byung Chul Jee[3], Chang Suk Suh[1,2,3,4], Hee Sun Kim[2], Sun Kyung Oh[2], Seok Hyun Kim[1,2] and Shin Yong Moon[1,2]

[1]Department of Obstetrics and Gynecology, College of Medicine, [2]Institute of Reproductive Medicine and Population, Medical Research Center, Seoul National University, Seoul, 110–744 and [3]Department of Obstetrics and Gynecology, Seoul National University Bundang Hospital, Seongnam, 463–707, Korea

[4]To whom correspondence should be addressed at: Department of Obstetrics and Gynecology, Seoul National University Bundang Hospital, 300 Gumi-dong, Bundang-gu, Seongnam, Kyunggi-do, 463–707, Korea. E-mail: suhcs@snu.ac.kr

BACKGROUND: An effective freezing–thawing technique is crucial for the clinical application of human embryonic stem (ES) cells. The aim of this study was to find an optimal cryopreservation protocol for human ES cells using slow freezing–rapid thawing without a programmable freezer. METHODS: The human ES cell line, SNUhES-3, was cultured on an STO feeder layer in gelatin-coated tissue culture dishes. All cryopreservation steps were performed using a simple commercial freezing container. The survival rate of cryopreserved–thawed human ES cells was estimated by counting colony numbers under a stereomicroscope. Initially, we compared the survival rates of cryopreserved human ES cells using three cryoprotectants: dimethylsulphoxide (DMSO), ethylene glycol (EG) and glycerol. In this experiment, 5% DMSO/95% fetal bovine serum (FBS) (vol/vol) showed the highest survival rate. We next tested the impact of various concentrations of FBS (95, 50 and 5%) with 5% DMSO, and then examined the effects of adding EG or glycerol to 5% DMSO + optimal FBS. RESULTS: No significant difference in survival rate was observed between 95 and 50% FBS in the presence of 5% DMSO. A significant improvement in survival rate was obtained by adding 10% EG to 5% DMSO + 50% FBS. After thawing, surviving cells were found to maintain the inherent characteristics of human ES cells. CONCLUSION: 5% DMSO + 50% FBS + 10% EG may be an optimal cryoprotectant for the slow freezing–rapid thawing of human ES cells.

Key words: cryopreservation/dimethylsulphoxide/ethylene glycol/human embryonic stem cells

Introduction

Embryonic stem (ES) cells are derived from the inner cell mass of blastocysts. These cells can be propagated indefinitely in an undifferentiated state *in vitro* and have the ability to differentiate into derivatives of all three embryonic germ layers, and into diverse cells and tissues. Evans and Kaufman (1981) first reported establishment of ES cells derived from mouse embryos, and subsequently several ES cell lines have been reported from sheep (1991), rabbit (1993), pig (1994), monkey (1995), avian (1996) and bovine (1996).

Recently, human ES cells were derived from human blastocysts (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000), and were found to be pluripotent and to have the ability to self-renew. These human ES cells are expected to have far-reaching applications in the areas of regenerative medicine, pharmacology, and basic scientific research (Reubinoff *et al.*, 2001). Human ES cells offer tremendous potential for clinical applications as an unlimited source of cells for transplantation and tissue regeneration. In addition, they offer the possibilities of studying basic developmental science and cell signalling.

In order to use human ES cells in research, satisfactory cryopreservation technologies are crucial. Moreover, efficient cryopreservation is essential for the establishment of a human ES cell bank (Gearhart, 1998). Several researchers have suggested that the characteristics of human ES cells may change during long-term culture (Andrews, 2004; Buzzard *et al.*, 2004). Therefore, the maintenance of early passage human ES cells is important, and it may be possible to preserve stocks of early passage cells by effective freezing and thawing.

Two methods are used to cryopreserve ES cell lines: slow freezing–rapid thawing (Whittingham *et al.*, 1972; Trounson and Mohr, 1983; Kaufman *et al.*, 1995) and vitrification (Rall and Fahy, 1985; Karlsson, 2002). The slow freezing–rapid thawing method has been commonly used to cryopreserve mouse ES cells (Robertson, 1987). Slow freezing protocols are straightforward and can be easily applied to the cryopreservation of ES cells or embryos. In addition, a large cell volume could be frozen in one vial. Moreover, when large volumes of cells are required for applications such as drug screening or clinical applications, slow freezing may be

beneficial. In addition, although these two methods are effective for murine ES cells (Kaufman *et al.*, 1995; Reubinoff *et al.*, 2001), it has been observed that the survivals of human ES cells subjected to slow freezing–rapid thawing are poor (Reubinoff *et al.*, 2001; Kim *et al.*, 2004). Sometimes, the conventional slow freezing method requires a programmable embryo freezing module designed to freeze mammalian embryos at a controlled rate of −0.4–0.6 °C/min (Shaw *et al.*, 1995). However, programmable freezers are relatively expensive, and not always available.

Vitrification is a simple cryopreservation method and has been widely used to cryopreserve mammalian embryos. Recently, some groups have reported on the cryopreservation efficiencies of human ES cells that have been exposed to modified vitrification procedures (Reubinoff *et al.*, 2001; Kim *et al.*, 2004; Richards *et al.*, 2004; Zhou *et al.*, 2004). However, vitrification is not an easy option because the steps required are more complicated than those of the slow freezing method. In addition, only small cell volume can be cryopreserved using this technique, and therefore it is unsuitable for clinical applications. Moreover, Reubinoff *et al.* (2001) reported that vitrified human ES cells showed considerable cell death and spontaneous differentiation after thawing or plating.

Thus in the present study, we undertook to develop a simple and convenient mass cryopreservation method using a freezing container instead of an expensive programmable freezer. Although combination of dimethylsulphoxide (DMSO) and ethylene glycol (EG) was shown to be efficient in vitrification of human ES cells (Reubinoff *et al.*, 2001) we examined the survival efficiency of human ES cells using various combinations of cryoprotectants, namely DMSO, EG and glycerol. During this work we tried to reduce the serum content based on the belief that it acts as a differentiation stimulator of human ES cells. The developed freezing method can cryopreserve large numbers of human ES cells using simple steps.

Materials and methods

Cell culture

STO feeder cell culture

The STO cell line was derived from a continuous line of SIM mouse embryonic fibroblasts (ATCC, CRL-1503, USA). The STO cell culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) containing 4 mmol/l L-glutamine, 1.5 g/l sodium bicarbonate, and 4.5 g/l glucose, supplemented with 10% fetal bovine serum (FBS; Hyclone, USA). STO cells were cultured in conditions of 5% CO_2, 37 °C. The culture medium was changed every other day. STO cells were grown as monolayers and propagated every 3 days after replating. The subcultivation ratio was 1:3 to 1:10. When ~80% of the cells were confluent, the culture medium was removed and replaced with mitomycin C solution. Cells were then incubated for 2.5 h except purposed stock cells. After mitotically inactivating STO cells, the mitomycin C solution was removed and the cells were washed with PBS. The cells were harvested using 0.25% (w/v) trypsin–0.53 mmol/l EDTA solution, neutralized with culture medium, and then centrifuged for 10 min at 1000 r.p.m. The cell freezing medium was 5% DMSO in complete growth medium, and cells were rapidly thawed in a 37 °C water bath. After removing freezing medium, cells were resuspended in growth medium, and replated on 0.1% gelatin-coated subculture dishes. Cells were incubated overnight, washed with STO cell culture medium, and then replaced with ES cell culture medium.

Human ES cell culture

The human ES cell line, SNUhES-3, was used to evaluate the efficiency of the mass cryopreservation of human ES cells. Human ES cells were cultured on an STO feeder layer in gelatin-coated tissue culture dishes. The culture medium consisted of DMEM and F-12 (1:1) supplemented with 20% Knock-out serum replacement (SR), 0.1 mmol/l β-mercaptoethanol, 1% non-essential amino acids, 50 IU/ml penicillin and 50 μg/ml streptomycin (all from Gibco, USA) and 0.4 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen, USA). The colonies were dissociated into several clumps by mechanical slicing using a glass pipette and subcultured on fresh STO feeder layers every 7 days.

Cryopreservation of human ES cells

Human ES cell colonies were dissected into several small clumps with a glass pipette 5 days after replating. After removing the culture medium by brief centrifugation, 100 clumps of human ES cells were transferred into a 2 ml cryo-vial (Sarstedt, Germany) containing 1 ml of pre-cooled (4 °C) freezing medium. The cryo-vials were then immediately placed into a freezing container ('Mr. Frosty'; Nalgene, USA), and cooled to −70 °C within a deep freezer. Although the temperature within the deep freezer dropped at a rate of −1 °C/min, the real cooling rate of the freezing container was −0.5 °C/min, as indicated in the manufacturer's manual. After keeping overnight, the cryo-vials were plunged into and stored in liquid nitrogen. One week later, the cryo-vials were rapidly thawed in a 37 °C water bath. The freezing medium was gradually diluted with 4 ml of ES culture medium, and then the human ES cell clumps were washed by gentle pipetting. After removing the suspension by brief centrifugation, ES culture medium was added to the clumps, which were then plated onto a fresh STO feeder layer. The growth medium was renewed on the 3rd day after thawing and then daily.

Several freezing media were prepared to compare survival rates. Initially, three cryoprotectants were compared; 5 and 10% dimethylsulphoxide (DMSO; Sigma, USA), 5% and 10% ethylene glycol (EG; Sigma, USA), or 5% glycerol (Sigma, USA). The mix was made up to 100% with FBS (vol/vol). In this experiment, 5% DMSO showed the highest survival rate in the presence of 95% FBS (Table I, Experiment I). Subsequently, the effects of different FBS concentrations were tested, i.e. 95, 50 or 5%; the mix was made up to 100% with DMEM/F-12, the basic human ES cell growth medium. After determining the optimal FBS content, we prepared freezing medium mixtures by adding either EG or glycerol to 5% DMSO + optimal FBS.

The survival rates of cryopreserved–thawed human ES cells were estimated by counting the number of developed colonies under a stereomicroscope 10 days after replating.

Characterization of cryopreserved human ES cells

Immunocytochemistry

Three passages after thawing, surface markers for human ES cells were defined. Colonies that had originated from cryopreserved–thawed human ES cells were fixed in 4-well culture dishes containing 4% paraformaldehyde for 30 min at room temperature. The primary antibodies used for immunocytochemistry were

Table I. The survival rates of human embryonic stem (ES) cells after slow freezing-rapid thawing using various medium compositions

Experiments	Cryoprotectant compositions	Fetal bovine serum (%)	*n*	Survival rate (%)
I	5% DMSO	95	6	11.3 ± 6.5[a]
	10% DMSO	90	6	1.3 ± 0.17[b]
	5% EG	95	6	0.3 ± 0.3[b]
	10% EG	90	6	0
	5% glycerol	95	6	0
II	5% DMSO	95	6	11.0 ± 6.9[c]
	5% DMSO	50	6	9.3 ± 3.9[d]
	5% DMSO	5	6	1.5 ± 0.1
III	5% DMSO +5% EG	50	6	4.7 ± 1.5[e]
	5% DMSO +10% EG	50	6	30.2 ± 1.6[f]
	5% DMSO +20% EG	50	6	0
	5% DMSO +5% glycerol	50	6	0
	5% DMSO +10% glycerol	50	6	0
	5% DMSO +20% glycerol	50	6	0

n = number of repeated experiments. Each experiment included 100 colonies of human ES cells.
Survival rates are represented as means ± SEM.
[a,b]$P < 0.05$, [c,d]not significant, [d,f]$P < 0.05$, [e,f]$P < 0.05$ (Mann–Whitney *U*-test, two-tailed).
DMSO = dimethylsulphoxide; EG = ethylene glycol.

stage-specific embryonic antigens (SSEA) and undifferentiated ES cell surface markers, i.e. SSEA-1, SSEA-3 and SSEA-4, and tumour rejection antigens (TRA)-1-60 and TRA-1-81 (all from Chemicon, USA). Antibody localization was detected by using Vectastain ABC reagent and a DAB kit (Vector Laboratories, Inc., USA).

Alkaline phosphatase (AP) activity was demonstrated at the same passages as above. Cryopreserved–thawed human ES cells were fixed in a 4-well culture dish using citrate–acetate formaldehyde fixative and incubated with FRV-alkaline, sodium nitrite and naphtol AS-BI alkaline solution mixture for 15 min at room temperature (Sigma, USA).

RT-PCR of Oct-4 and Nanog

Total RNA was extracted using a QIAGEN RNeasy kit (QIAGEN, USA). Standard reverse transcription reactions were performed with 300 ng of total RNA with random hexamers using SuperScript™ First-Strand Synthesis System (Invitrogen, USA). The PCR was performed using a rtaq PCR master mix (TaKaRa, Japan). Primer sequences are shown in Table II. After incubation for 5 min and initial denaturation for 30 s at 94 °C, 30 amplification cycles were performed (annealing for 30 s at 61 °C (Oct-4) or 56 °C (Nanog), extension for 30 s at 72 °C), and this was followed by a final extension for 7 min at 72 °C. Successful PCR was confirmed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

In vitro differentiation of cryopreserved–thawed human ES cells

The *in vitro* differentiation of cryopreserved–thawed human ES cells was induced in suspension cultures and the formation of embryoid bodies (EB) was confirmed later. Cryopreserved–thawed human ES cells were cultured for three passages in ES culture medium excluding bFGF, and EB formation was induced in bacteriological dishes by treating with collagenase (200 IU/ml) (Gibco, USA) or by mechanical dissection. After overnight culture in suspension, ES cells formed floating aggregates. EB could be maintained in suspension for 31 days.

The differentiation potentials of cryopreserved human ES cells were confirmed by RT–PCR. Total RNA extraction, cDNA synthesis and PCR were carried out using the conditions previously described for the RT–PCR of Oct-4 and Nanog. To detect the differentiation potential of EB to the three germ layers, α-fetoprotein (AFP), Brachyury and PAX-6 primer were used as representative markers of the three embryonic germ layers, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard (Table II).

Statistical analysis

The Mann–Whitney *U*-test in SPSS for Windows (version 10.0) was used to compare means, and the χ^2-test was used to compare proportions. $P < 0.05$ (two-tailed) was considered statistically significant.

Results

Analysis of the efficiency of the developed cryopreservation method in various medium compositions

Impact of different FBS concentrations

The concentration of DMSO was fixed at 5%, and three different FBS concentrations were compared: 95, 50 and 5%. As the FBS content decreased, the survival rates of cryopreserved cells also decreased. However, no significant difference was observed between 95 and 50% FBS in the presence of 5% DMSO (Table I, Experiment II). These results indicate that the optimal content of FBS may be ≥50%. However, serum contains many unknown growth factors or cytokines that can induce human ES cell differentiation. Therefore, we chose 50% FBS for the next experiment.

Impact on survival rate of adding EG or glycerol to 5% DMSO + 50% FBS

EG has been successfully used as a cryoprotectant for the vitrification of mammalian embryos or ES cells. However, the majority of previous studies have used only EG for vitrification. Thus, we examined the uses of EG or glycerol for the slow freezing of human ES cells. At 10 days after thaw-

Table II. RT–PCR primers used to detect gene expressions in human embryonic stem cells

Gene	Primer sequence	Product size (bp)	Category
Oct-4	F: GGCGTTCTCTTTGGAAAGGTGTTC R: CTCGAACCACATCCTTCTCT	314	Stem cell marker
Nanog	F: TGCCTCACACGGAGACTGTC R: TGCTATTCTTCGGCCAGTTG	390	Stem cell marker
α-Fetoprotein	F: AGAACCTGTCACAGCTGTG R: GACAGCAAGCTGAGGATGTC	676	Endoderm
Brachyury	F: TAAGGTGGATCTTCAGGTAGC R: CATCTCATTGGTGAGCTCCCT	252	Mesoderm
PAX-6	F: AACAGACACAGCCCTCACAAACA R: CGGGAACTTGAACTGGAACTGAC	275	Ectoderm
GAPDH	F: AGCCACATCGCTCAGACACC R: GTACTCAGCGGCCAGCATCG	302	Internal standard

ing, the mean survival rate was significantly higher for 5% DMSO + 50% FBS + 10% EG compared to other combinations (Table I, Experiment III). The rate was also significantly higher than that in 5% DMSO + 50% FBS. Moreover, the survival rates obtained for 5% DMSO + 50% FBS + 10% EG were rather consistent at: 31, 25, 31, 26, 34 and 34%. In contrast, no colonies were observed after adding 20% EG or 5–20% glycerol to 5% DMSO + 50% FBS.

Characterization of cryopreserved–thawed human ES cells

In vitro colony morphology of human ES cells

Three days after thawing, human ES cells started to form small colonies, and at 10 days after thawing, the sizes of the colonies were similar to day 7 human ES cell colonies that had not been frozen (Figure 1).

Expression of embryonic stem cell markers

To confirm that the cryopreserved human ES cells retained the key properties and characteristics of human pluripotent ES cells, several markers were examined for up to three passages after thawing. Cryopreserved human ES cells showed alkaline phosphatase activity (Figure 2A), and immunostaining for anti-SSEA-1 was negative and for anti-SSEA-3 was partially positive; these results were similar to those of unfrozen human ES cells. Anti-SSEA-4, TRA-1-60 and TRA-1-81 were also expressed in cryopreserved human ES cells (Figure 2B–F).

Oct-4 and Nanog mRNA, representative markers of undifferentiated human ES cells, were clearly detected in cryopreserved–thawed human ES cells at similar expression levels (Figure 3).

In vitro differentiation potential of cryopreserved–thawed human ES cells

Human EB were spontaneously induced under standard culture conditions. The EB showed cystic or a simple round morphology (Figure 4), and gene expressions indicative of three embryonic germ layers were observed (Figure 3). This means that the human EB in our experiment spontaneously differentiated into three embryonic germ layers. These cells expressed the representative marker genes of three embryonic germ layers, namely, α-fetoprotein (AFP; endoderm), Brachyury (mesoderm), and PAX-6 (ectoderm). These genes were only expressed in EB, and not in ES cells, which indicates that human EB can differentiate into all three germ layers. However, human ES cells maintained an undifferentiated state whether cryopreserved–thawed or not.

Discussion

The object of this study was to develop a slow freezing–rapid thawing protocol by modifying cryoprotectant make-up without using a programmable freezer. This approach may enable the cryopreservation of large numbers of human ES

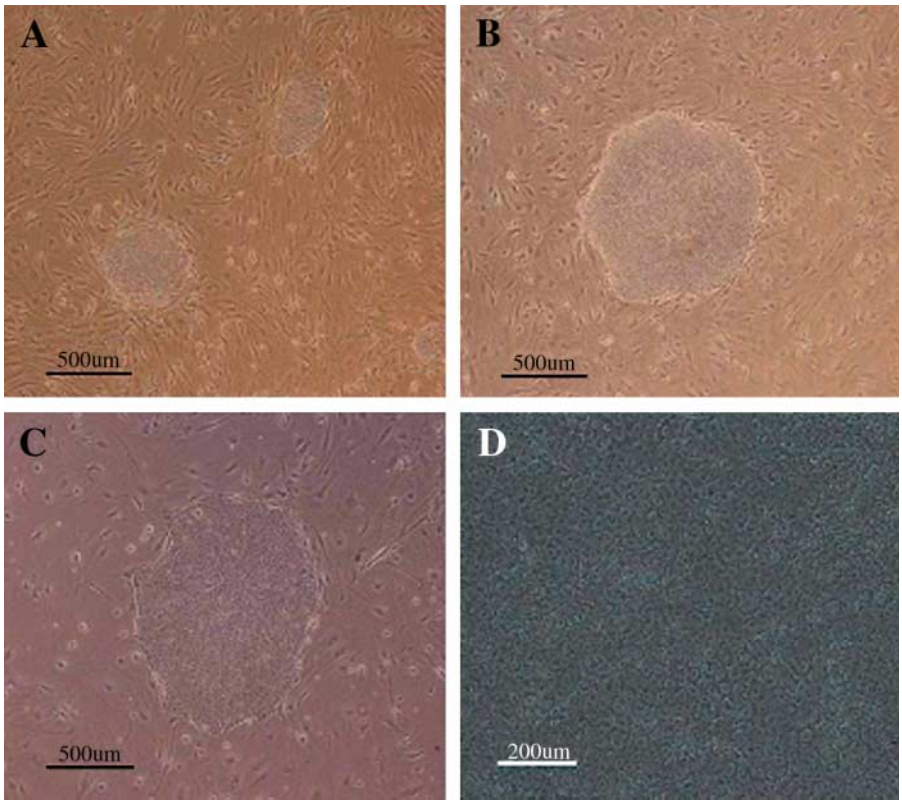

Figure 1. Cryopreserved human embryonic stem (ES) cells showed a normal ES cell morphology at: (**A**) 5 days, (**B**) 7 days, and (**C**) 10 days after thawing. The colony sizes observed at 10 days after thawing were similar to those of the control ES cells at 7 days (the cryopreserved cells started to propagate 3 days after thawing). (**D**) High magnification view of **C**.

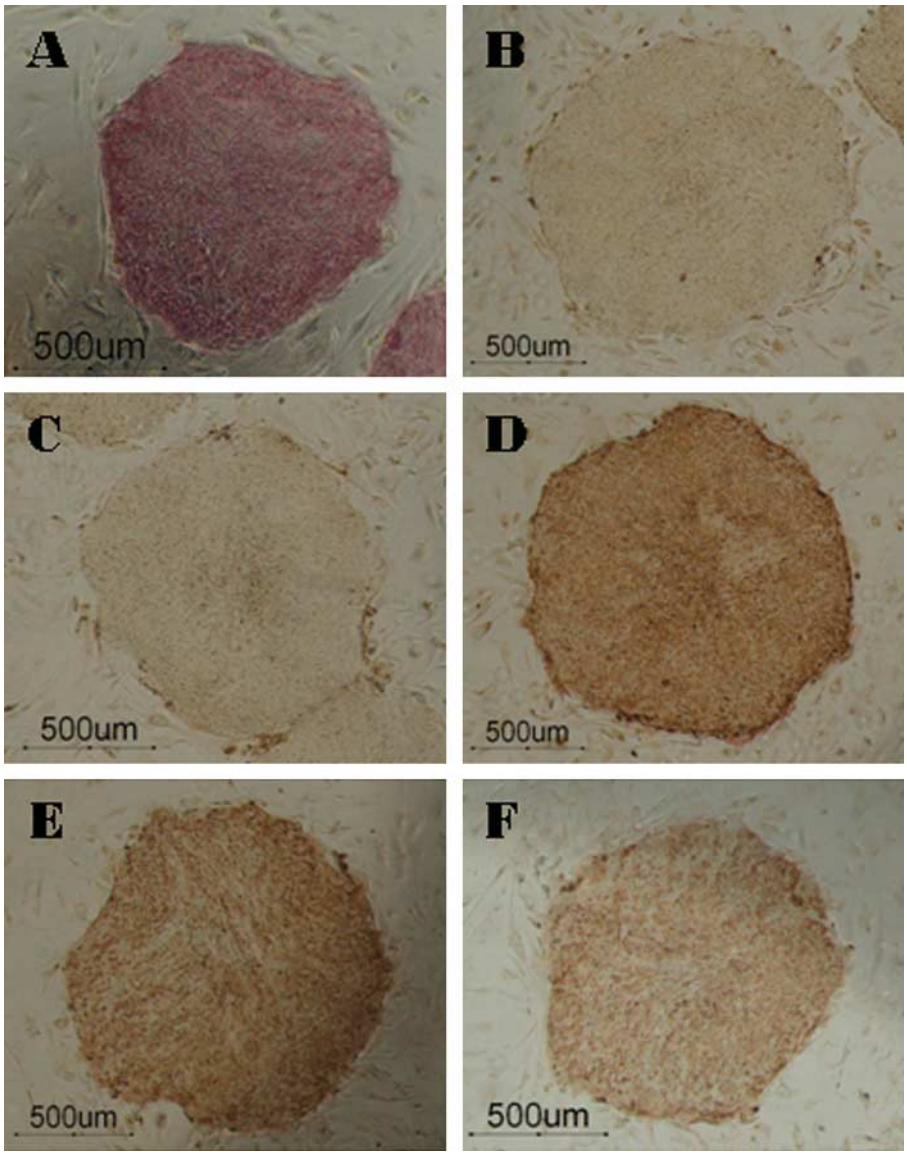

Figure 2. Marker expressions of cryopreserved human embryonic stem (ES) cells: (**A**) cells were positive for alkaline phosphatase expression, (**B**) negative for anti-stage-specific embryonic antigen (SSEA)-1 expression, (**C**) partially positive for anti-SSEA-3 expression, and positive for (**D**) anti-SSEA-4, (**E**) anti-tumour rejection antigen (TRA)-1-60, and (**F**) anti-TRA-1-81 expression.

cells in one vial without the need for a programmable freezer or direct contact with liquid nitrogen.

We compared the survival rates of the cells treated with DMSO, EG and glycerol containing basic cryoprotectants. In our experiment, DMSO showed the highest survival rate, which is consistent with other reports concerning the superiority of DMSO (Chen *et al.*, 2001; Chi *et al.*, 2002). Furthermore, 5% DMSO showed a better survival rate compared with 10% DMSO, which has been commonly used in slow freezing protocols of human ES cells.

Serum is a complex mixture and probably contains various compounds that are beneficial and detrimental to human ES cells. Moreover, different serum batches vary widely in their ability to support the vigorous undifferentiated proliferation of human ES cells. Thus, replacing serum with defined components should reduce the experimental variability associated with serum batch variations (Amit *et al.*, 2000). To maintain the undifferentiated state of human ES cells, most have used SR instead of serum (Chiu and Rao, 2003). In our experiment, we reduced the content of FBS from 95% to 50% or 5%. In this trial, 95% FBS showed the highest survival rate. However, no significant difference was observed between the survival rates of 95% FBS- and 50% FBS-treated cells. Serum is essentially needed for the cryopreservation of human ES cells, and freezing ES cells without FBS is associated with a poor survival rate. According to our results, the optimal content of FBS may be $\geq 50\%$, and here we considered 50% FBS as sufficient for human ES cell cryopreservation.

Ethylene glycol, a rapid permeable cryoprotectant, appears to have a low toxic effect on mice and human embryos (Ali and Shelton, 1993; Chi *et al.*, 2002). It is also used in human ES cell vitrification solutions combined with DMSO (Reubinoff *et al.*, 2001). In our experiment, an improved

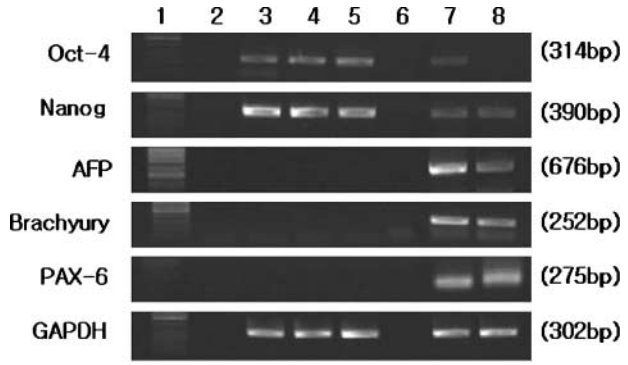

Figure 3. RT–PCR analysis of gene expressions related to the non-differentiation and the differentiation of cryopreserved human embryonic stem (ES) cells and human embryoid bodies (EB); Oct-4 and Nanog mRNA markers of the undifferentiated state in ES cells; and α-fetoprotein (AFP) (endoderm), Brachyury (mesoderm) and PAX-6 (ectoderm) as markers of the three embryonic germ layers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. Lane 1: 50 bp DNA ladder; lane 2: ES, RT; lane 3: human ES cells (passage 91); lane 4: human ES cells (passage 131); lane 5: cryopreserved–thawed human ES cells (passage 117); lane 6: EB, RT; lane 7: human EB, from control human ES cells (day 31); lane 8: human EB; from cryopreserved–thawed human ES cells (day 31).

survival rate was obtained only after adding 10% EG to freezing medium containing 5% DMSO + 50% FBS. Mean survival rate increased 3-fold versus 5% DMSO + 50% FBS and this was consistently achieved. This finding indicates that cryopreservation efficiency was both remarkably increased and stabilized by adding 10% EG. Since equimolar combinations of DMSO and EG have been commonly used in vitrification of human ES cells, we also tested the efficiency of 5% DMSO combined with 5% EG. However, an improvement was not observed in survival rate. Furthermore, no colonies were observed after adding 5–20% glycerol to 5% DMSO.

We concluded that 5% DMSO with 10% EG is a more effective cryoprotectant for slow freezing of human ES cells even at 50% FBS levels. It is not certain why 5% DMSO with 10% EG is superior to 5% DMSO alone or in other combinations. It is likely that EG may work efficiently with a lower level of DMSO and FBS in slow freezing methods using a freezing container.

A recent report indicates that loading human ES cells with the disaccharide trehalose prior to cryopreserving in a DMSO-containing cryoprotectant solution improves cell viability, although the cells adherent to, or embedded in, a Matrigel matrix are cultured and cryopreserved (Ji *et al.*, 2004). Trehalose is a high mol. wt sugar and one of the non-penetrating cryoprotectants. The use of trehalose could reduce the toxic effect of DMSO by dehydrating cells. It will be further investigated whether the treatment of trehalose could be useful in the slow freezing method of human ES cells.

In summary, we applied slow freezing to cryopreserve human ES cells using a mixture of cryoprotectants without using a programmable freezer. Our results indicate that the addition of 10% EG to 5% DMSO + 50% FBS produced satisfactory survival rates using our developed technique for human ES cells. Our results also show that the mass

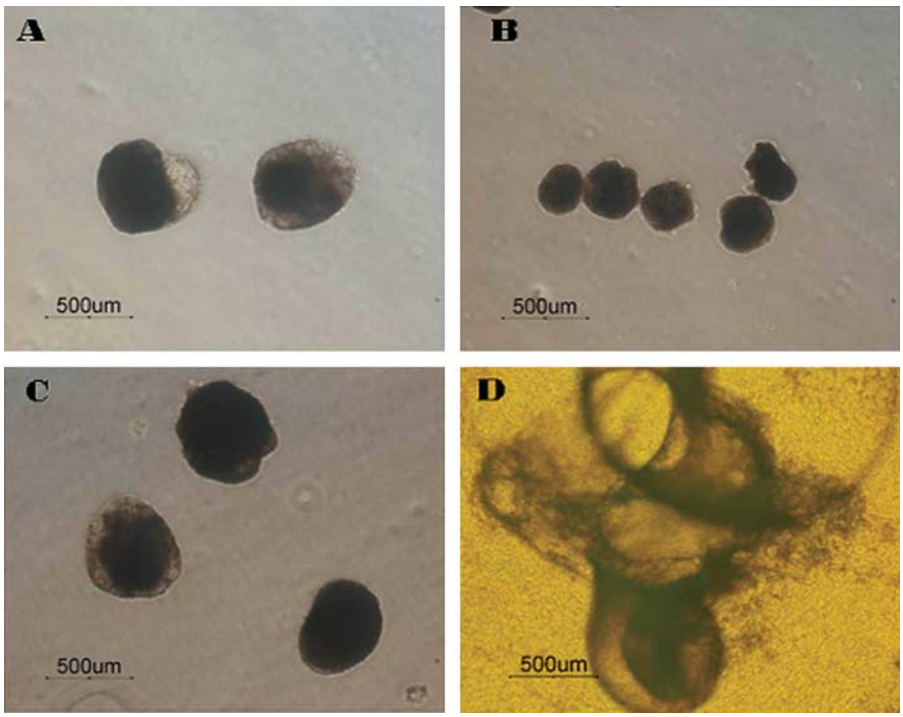

Figure 4. Cryopreserved human embryonic stem (ES) cells differentiated spontaneously to embryoid bodies (EB). These EB aggregates induced the differentiation of ES cells into the three embryonic germ layers. It was not established whether cystic EB can differentiate faster than simple EB, but cystic EB showed variable cell types after attachment. (**A**) Cystic human EB, (**B**) 14 day old human EB, (**C**) 21 day old human EB, (**D**) photomicrograph of human EB spreading during attached culture.

cryopreservation of human ES cells may be possible without the cells contacting liquid nitrogen directly. The key properties of human ES cells, namely, proliferative ability and pluripotency, were maintained after cryopreservation using the developed method. Since this method is similar to those used for other mammalian cells, it can be easily performed in many laboratory settings. The method could be used to store stocks of early ES cell passages, and transfer cells to other laboratories, and thus we hope that it contributes to the widespread use of human ES cell lines.

Acknowledgement

This study was supported by a grant (code: SC11011) from the Stem Cell Research Center of 21st Century Frontier research Program, the Ministry of Science and Technology, Republic of Korea.

This study was also supported by the research fund of the College of Medicine, Seoul National University (Grant No. 8002002116).

References

Ali J and Shelton N (1993) Vitrification of preimplantation stage of mouse embryos. J Reprod Fertil 98,459–465.

Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, Waknitz MA, Itskovitz-Eldor J and Thomson JA (2000) Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol 227,271–278.

Andrews PW (2004) Response: Karyotype of human ES cells during extended culture. Nat Biotechnol 22,382.

Buzzard JJ, Gough NM, Crook JM and Colman A (2004) Karyotype of human ES cells during extended culture. Nat Biotechnol 22,381–382.

Chen SU, Ho HN and Yang YS (2001) Vitrification of embryos and oocytes with 5.5 mol/l ethylene glycol and 1.0 mol/l sucrose. Hum Reprod 16, 1778–1779.

Chi HJ, Koo JJ, Kim MY, Joo JY, Chang SS and Chung KS (2002) Cryopreservation of human embryos using ethylene glycol in controlled slow freezing. Hum Reprod 17,2146–2151.

Chiu AY and Rao MS (2003) Human embryonic stem cells. Humana Press pp,113–126.

Evans MJ and Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292,154–156.

Gearhart J (1998) New potential for human embryonic stem cells. Science 282,1061–1062.

Ji L, de Pablo JJ and Palecek SP (2004) Cryopreservation of adherent human embryonic stem cells. Biotechnol Bioengng 88,299–312.

Karlsson OM (2002) Cryopreservation: freezing and vitrification. Science 296,655–656.

Kaufman RA, Ménézo Y, Hazout A, Nicollet B, DuMont M and Servy EJ (1995) Cocultured blastocyst cryopreservation: experience of more than 500 transfer cycles. Fertil Steril 64,1125–1129.

Kim EY, Park SY, Yoon JY, Ghil GS, Lee CH, Lee GS, Tae JC, Kim NH, Lee WD, Chung KS et al. (2004) A new efficient cryopreservation of human embryonic stem cells by a minimum volume cooling method. Kor J Fertil Steril 31,41–50.

Rall WF and Fahy GM (1985) Ice-free cryopreservation of mouse embryos at −196 °C by vitrification. Nature 313,573–575.

Reubinoff BE, Pera MF, Fong CY, Trounson AO and Bongso A (2000) Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 18,399–405.

Reubinoff BE, Pera MF, Vajta G and Trounson AO (2001) Effective cryopreservation of human embryonic stem cells by open pulled straw vitrification method. Hum Reprod 16,2187–2194.

Richards M, Fong CY, Tan S, Chan WK and Bongso A (2004) An efficient and safe xeno-free cryopreservation method for the storage of human embryonic stem cells. Stem Cells 22,779–789.

Robertson EJ (1987) Embryo derived stem cell lines. In Robertson EJ (ed.) Teratocarcinomas and Embryonic Stem Cells: A Practical Approach. IRL Press, Oxford, pp. 71–112.

Shaw JM, Ward C and Trounson AO (1995) Evaluation of propanediol, ethylene glycol, sucrose and antifreeze proteins on the survival of slow-cooled mouse pronuclear and 4-cell embryo. Hum Reprod 10,396–402.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS and Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282,1145–1147.

Trounson AO and Mohr L (1983) Human pregnancy following cryopreservation thawing and transfer of an eight-cell embryo. Nature 303,707–709.

Whittingham DG, Leibo SP and Mazur P (1972) Survival of mouse embryos frozen to −196 °C and −269 °C. Science 178,411–414.

Zhou CQ, Mai QY, Li T and Zhuang GL (2004) Cryopreservation of human embryonic stem cells by vitrification. Chin Med J 117,1050–1055.

Submitted on January 15, 2005; resubmitted on February 11, 2005; accepted on February 18, 2005