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Improving the Survival and Maintenance of the Undifferentiated State of Cryopreserved Human Embryonic Stem Cells by Extended Incubation with Feeder Cells Overnight before Vitrification

Soo Kyung Cha, Kyoung Hee Choi, Ju-Mi Shin, Kyu-Hyung Park[1], Tae Ki Yoon Hyung Min Chung[1] and Dong Ryul Lee†

Fertility Center of CHA General Hospital, CHA Research Institute, Pochon CHA University College of Medicine, Seoul 135-081, Korea

[1]CHA Stem Cell Institute, Pochon CHA University College of Medicine, Seoul 135-081, Korea.

동결에 앞서 시행된 지지세포와의 추가 공배양이 인간 배아줄기세포의 유리화 동결보존 후 생존율과 미분화 유지에 미치는 영향

차수경 · 최경희 · 신주미 · 박규형[1] · 윤태기 · 정형민[1] · 이동률†

포천중문의과대학교 차병원 여성의학연구소, [1]포천중문의과대학교 줄기세포연구소

ABSTRACT : This study was conducted to develop an efficient cryopreservation method of human embryonic stem (ES) cells using vitrification. In an initial experiment, sub-clumps of human ES cells (CHA-hES3 and CHA-hES4) were vitrified using grids after incubation with STO feeder cells for 1 or 16 h (Groups 1-1 and 1-2, respectively). After storage for 2~4 months, thawed clumps were re-plated on a fresh feeder layer. The survival rates of warmed CHA-hES3 and CHA-hES4 cells of Group 1-2 were significantly higher than those of the corresponding Group 1-1 cells. In the second experiment, human ES cells were vitrified after incubation with feeder or feeder-conditioned medium (Groups 2-1 to -7). Relative mRNA expression of BM proteins and survival rates were increased following incubation of ES cells with fresh feeder cells for 16 h. In conclusion, increasing of tight adhesion between ES cells by extended incubation with feeder could reduce cryoinjury after vitrifying/warming.

Key words : Human embryonic stem cells, Vitrification, Extended incubation with STO feeder cells, Survival rate.

INTRODUCTION

Human embryonic stem (ES) cells are derived from transferring the inner cell mass (ICM) of the blastocyst into a plastic laboratory culture dish that contains nutrients, growth factors, and feeder cells. Stem cells are capable of unlimited and undifferentiated proliferation, and remain karyotypically normal and phenotypically stable. Moreover, ES cells have the ability to differentiate into a wide variety of cell types *in vitro* and *in vivo* (Thomson et al., 1998; Reubinoff et al., 2000).

Successful storage is very important for the maintenance, manipulation, and widespread use of stem cells. While several attempts have resulted in the improvement of outcome (Reubinoff et al., 2001), cryopreservation of human ES cells is still limited because of technical difficulty or the properties of the cell. In fact, survival rates are still obviously variable depending to researcher's skill and experience, as well as the condition of ES cell. So, the development of a simple and efficient method will be needed for routine application.

Recent reports indicate that soluble factors and extra-

† Correspondence: Fertility Center of CHA General Hospital, CHA Research Institute, Pochon CHA University College of Medicine, 606-5 Yeoksam-dong, Gangnam-gu, Seoul 135-081, Korea, Tel: 82-2-3468-3421, Fax: 82-2-501-8704, E-mail: drleedr@cha.ac.kr

cellular matrix (ECM) produced by feeders may be important for proliferating and maintaining human ES cells (Xu et al., 2001; Richards et al., 2002; Richards et al., 2003). ECM provides a platform for multiple signaling mechanisms, which may explain its importance in cell differentiation, migration, and survival (Li et al., 2001). The basement membrane (BM) contains many molecules, including the structural proteins, type IV collagen and laminin, which are essential for this process. However, the effects of feeder-producing factors (FPF) on the expression of specific BM proteins and survival of hES cells after cryopreservation are currently unclear. In this study, we evaluate the effects of extended incubation with feeder cells on expression of BM proteins in sub-clumps of human ES cells, and cell survival after vitrification and warming.

MATERIALS AND METHODS

1. Maintenance of Human ES Cells

The STO cell line, purchased from American Type Culture Collection, was sub-cultured in Dulbecco's modified Eagle medium (DMEM) High Glucose (Invitrogen, Grand Island, NY) supplemented with 2 mM glutamine (Sigma), 0.1 mM β-mercaptoethanol (Sigma), and 1% nonessential amino acids (Invitrogen, Grand Island, NY). Next, STO cells were treated with 10 μg/mL mitomycin C (Sigma) for 1.5 h, extensively washed in PBS, and re-plated at 60,000 cells/cm^2 in gelatin-coated tissue culture dishes.

Two lines (CHA-hESC3 and CHA-hESC4, Pochon CHA University, Korea) were used for this study (Ahn et al., 2006; Han et al., 2008). ES cell colonies were cultured in DMEM/F12 high-glucose medium without pyruvate, supplemented with 20% serum replacement (SR), 0.1 mM β-mercaptoethanol, 1% nonessential amino acids, 1 mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 4 ng/mL basic fibroblast growth factor (bFGF, Invitrogen). The cells were found to maintained a normal karyotype (XY), as well being shown to be positive for Nanog, Oct-4, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and alkaline phosphatase (AP), while being negative for SSEA-1 (data not shown).

Experiment 1. Survival of vitrified/warmed human ES cells, based on incubation times on the STO feeder cell layer

ES cell lines (CHA-hESC3: 40~60 passages; CHA-hESC4: 30~40 passages) were cultured on a feeder layer of mitotically inactivated mouse STO cells in a gelatin-coated 4-well culture dish. Cultured colonies were mechanically dissected into 2~3 small clumps, and re-plated onto a new feeder layer for maintenance. For cryopreservation using vitrification, colonies with approximately 500~1,000 undifferentiated human ES cells were mechanically isolated under a stereomicroscope, and divided into two groups. One group was incubated in ES medium for 1 h before vitrification (Group 1-1). The other was incubated in ES medium containing new STO feeder cells for 16 h (Group 1-2). After incubation, colonies were pre-equilibrated for 2.5 min in 2 mL of Dulbecco's phosphate-buffered saline (DPBS, Invitrogen) supplemented with 1.5 M ethylene glycol (EG, E-9129; Sigma) and 10% (v/v) fetal bovine serum (FBS, Invitrogen) at 37℃, and subjected to final equilibration in the same volume of DPBS supplemented with 5.5 M EG, 1.0 M sucrose, and 10% FBS for 20 sec. Five to ten colony clumps were loaded on an electron microscope copper grid (Gilder, Westchester, PA) using a fine glass pipette. Excess cryoprotectant solution was removed with underlying sterilized paper (Kimwipes, Yuhan-Kimberly, Gunpo, Korea). The grids containing ES cell clumps were immediately plunged into liquid nitrogen (LN_2). For long-term storage, a cryovial cap and goblet were used for grid placement (Yoon et al., 2003).

After storing for 2~4 months, thawed clumps were re-plated on a fresh feeder layer, and the survival and spontaneous differentiation rates of vitrified-warmed ES cells were observed. For warming, grids were sequentially trans-

ferred to culture dishes containing 2 mL of DPBS supplemented with 1.0, 0.5, 0.25, 0.125, or 0 M sucrose and 10% (v/v) FBS at intervals of 2.5 min at 37℃. ES clumps were detached from grids by gentle blowing of the medium, and transferred to new ES medium with STO feeder cells. After culture for 5~6 days, the survival and spontaneous differentiation of colonies was observed under a phase-contrast microscope.

Experiment 2. BM expression of human ES cells and survival after vitrification/warming, based on incubation with STO cells or STO-conditioned medium

The CHA-hES3 line was used for this experiment. Cultured human ES cells were mechanically detached from culture dishes, and incubated in 7 systems, specifically, Group 2-1: naive ES cells (no incubation); Group 2-2: Incubation for 1 h in ES medium; Group 2-3: Incubation for 1 h in ES medium with new STO cells; Group 2-4: Incubation for 16 h in ES medium with new STO cells (1 day after preparation); Group 2-5: Incubation for 16 h in ES medium with old STO cells (5 days after preparation); Group 2-6: Incubation for 16 h in ES medium with new STO cell-conditioned medium (CM); and Group 2-7: Incubation for 16 h in ES medium with old CM. After incubation, ES clumps were divided into 2 fractions. One was sampled for molecular markers and ECM expression, while the other was vitrified and warmed for the analysis of cell survival.

For quantifying the relative expression of ECM, RNA isolation and RT-PCR were performed, as described above. After the initial reaction, samples were either directly used for real-time PCR for selected genes or stored at −20℃. Oct-4 (Forward: 5'-gga aag gct tcc ccc tca ggg aaa gg-3', Reverse: 5'-aag aac atg tgt aag ctg cgg ccc-3': 460 bp, GenBank Accession Number NM 002701), Rex-1 mRNA (Forward: 5'-ctg aag aaa cgg gca aag ac-3', Reverse: 5'-gaa cat tca agg gag ctt gc-3': 343 bp, GenBank Accession Number NM 174900), Nidogen mRNA (Forward: 5'-agg gtg tct ggg tgt ttg ag-3', Reverse: cga gca ctg gtg tct gtt gt-3': 421 bp, GenBank Accession Number NM 002508), Fibronectin 1 mRNA (Forward: 5'-aag gtt cgg gaa gag gtt gt-3', Reverse: 5'-tgg cac cga gat att cct tc-3': 402 bp, GenBank Accession Number NM 002026), Perlecan (Forward: 5'-gct att ctg gct tgt cct gc-3', Reverse: 5'-cgg gta ctc agg tgg aaa ga-3': 521 bp, GenBank Accession Number NM 005529), Laminin 1 (Forward: 5'-act gaa gta cag cgt ggc ct-3' , Reverse: 5'-gtt cag aca ctt ccc ggt gt-3': 555 bp, GenBank Accession Number NM 005559) and Collagen type IV (Forward: 5'-ggg ct acct gga gaa aaa gg-3', Reverse: 5'-tcc tgg aga gcc acc aat ac-3': 563 bp, GenBank Accession Number NM 001845) levels were quantified using semi-quantitative or real-time RT-PCR, relative to steady-state ribosomal protein (18S) mRNA (Forward: 5'-tac cta cct ggt tga tcc tg-3', Reverse: 5'-ggg ttg gtt ttg atc tga ta-3': 255 bp, GenBank Accession Number K03432), following reverse transcription of total RNA. Complementary DNA quantification was performed on a DNA Engine 2 fluorescence detection system (MJ research) using the DyNAmo SYBR green qPCR kit (Finnzymes). Reactions were performed in a final volume of 20 μL containing 4 μL DEPC-treated water, 2 μL forward primer (5 pmol), 2 μL reverse primer (2 pmol), 10 μL premix with SYBR Green, and 2 μL of cDNA template. The PCR protocol employed an initial denaturing step at 95℃ for 10 min, followed by 45 cycles of denaturation for 30 sec at 95℃, annealing of primers at 58~62℃ for 30 sec, and extension at 72℃ for 30 sec. Fluorescence was measured at the end of each cycle at 72℃. The final step was the generation of a melting curve in which the temperature was raised from 65℃ to 95℃ at 0.1℃/sec with constant monitoring of fluorescence, followed by a cooling stage of 40℃ for 30 sec. The relative quantification of gene expression was analyzed using the $2^{-\triangle\triangle CT}$ method [11].

2. Characterization of Human ES Cells after Vitrification and Warming

After confirming the survival of human ES cells, colo-

nies were maintained and sub-cultured every 5~6 days. Surviving ES cells were characterized by molecular and immunological marker expression, as well as teratoma formation. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to assess the expression of ES-specific marker genes in colonies, such as Oct-4, Nanog, Rex-1, and Sox-2. The following genes were amplified using primers specified in parentheses: Oct-4 (Forward: 5'-cgt tct ctt tgg aaa ggt gtt c-3', 5'-aca ctc gga cca cgt ctt tc-3': 250 bp, GenBank Accession Number AJ 297527), Nanog (Forward: 5'- gct tgc ctt gct ttg aag ca-3', 5'-ttc ttg act ggg acc ttg tc-3': 340 bp, GenBank Accession Number NM 024865), Rex-1 (Forward: 5'-ctg aag aaa cgg gca aag ac-3', 5'-gaa cat tca agg gag ctt gc-3': 302 bp, GenBank Accession Number AF 450454), Sox-2 (Forward: 5'-aga acc cca aga tgc aca ac-3', 5'-ggg cag cgt gta ctt atc ct-3': 220 bp, GenBank Accession Number NM 003106), and GAPDH (Forward: 5'- ttt tgg ctc ccc cct gca aat-3', Reverse: 5'-ctt tta act ctg gta aag tgg-3': 310 bp, GenBank Accession Number M33197). The PCR conditions employed included denaturation at 94℃ for 5 min, 30~35 cycles of 30 sec at 94℃, 30 sec at 55~60℃, and 30 sec at 72℃. A final extension step for 10 min at 72℃ completed the amplification reaction, after which products were separated by 1.5% agarose gel electrophoresis, and verified by automated nucleotide sequencing. The negative controls included mock transcription without RNA or PCR with distilled deionized water.

Human ES cells were fixed in 4% paraformaldehyde in Dulbecco's PBS (Gibco) for nucleus counting using 1 μg/mL 4',6'-diamidino 2-phenyindiol (DAPI, Sigma), and detection of alkaline phosphatase activity. For visualization, a mixture of violet chromogen BCIP (Sigma) and NBT (Sigma) was added to the alkaline phosphatase reaction buffer. For immunocytochemical analysis of surface markers, fixed colonies were incubated overnight with MC480 (SSEA-1), MC813 (SSEA-4), TRA-1-60, TRA-1-81 at 1:100~500 dilution, and visualized with FITC or Cy3-conjugated secondary antibody (Zymed).

To determine pluripotency, 100 to 200 ES colonies were harvested and injected with a sterile 26G needle into testicular capsules of 4- to 8-week-old severe combined immunodeficient (SCID) mice (CB 17 strain; Jackson Laboratory, Bar Harbor, ME). After 10 to 12 weeks, the resulting tumors were fixed in 10% neutral buffered formalin, embedded in paraffin, and examined histologically by staining with hematoxylin and eosin.

3. Cytogenetic Analysis of Thawed Human ES Cells

Thawed ES colonies were transferred mechanically to plastic Petri dishes to induce embryoid body (EB) formation, and incubated for 7 days in ES culture medium without bFGF. Well-formed EB (5~10) were transferred onto a fibronectin-coated coverslip, and cultured for another 24 h. Cells were incubated in culture medium with 0.1 μg/mL of colcemid for 3~4 h, and fixed in methanol/acetic acid (3:1, v/v). Following Giemsa staining, a cytogenetic specialist examined the karyotype of chromosomes at a resolution of 300 bands. At least 50 cells were examined from each sample.

4. Statistical Analysis

For statistical comparison, survival and differentiation rates were analyzed using the Chi-square test and gene expression of ECM with one-way ANOVA in the Statistical Analysis System. $P<0.05$ was considered statistically significant.

RESULTS

1. Effects of Incubation Time on STO Feeder Cells on Survival and Spontaneous Differentiation of Human ES Cells after Vitrification/Warming

We assessed ES cell survival at the formation of an undifferentiated colony after 5~6 days of re-plating. Moreover, spontaneous differentiation of ES cells was identified when 50% cells in the colony were morphologically altered within 3 passages. The Group 1-1 colony (incubation for

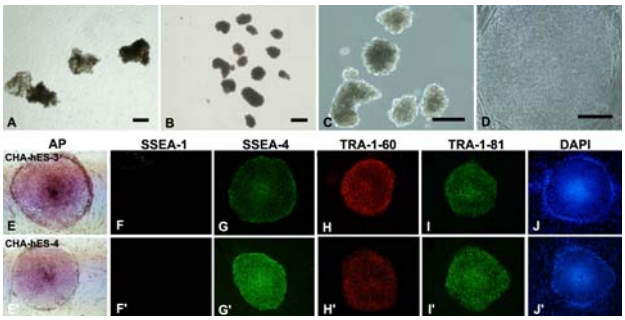

Fig. 1. Morphological features of human ES cells after incubation with STO feeder cells for 1 h (A) or 16 h (B-C). (D) Morphology of re-plated human ES cells grown on STO feeder after vitrifying/warming. (E-J) Marker expression in re-plated CHA-hES3 and −4 cells vitrified/warmed after incubation with STO feeder cells for 16 h. E, alkaline phosphatase staining. F, SSEA-1. G, SSEA-4. H, TRA-1-60. I, TRA-1-81, and J, DAPI. Bars indicate 100 μm.

1 h after mechanical detachment from the culture dish) was slightly aggregated (Fig. 1A). However, Group 1-2 cells (incubation for 16 h after mechanical detachment from culture dish) were tightly compact and attached slightly on the new feeder (Fig. 1B, C). The survival rates of warmed CHA-hESC3 and CHA-hESC4 in Group 1-2 were significantly higher than those in Group 1-1 (80.0%, 83.3% vs. 40.0%, 44.4%, $P<0.05$). Moreover, spontaneous differentiation rates in Group 1-2 were lower than those in Group 1-1 (6.8%, 5.3% vs. 33.3%, 30.0%, $P<0.05$, Table 1). Vitrified/warmed human ES cells remained undifferentiated (Fig. 1D), and maintained normal diploid karyotypes (46-XY) throughout extended culture over more than 60 passages (data not shown).

For characterization of thawed ES colonies from CHA-hES3 and 4 were fully characterized by AP staining, immunostaining of several antibodies against cell-surface carbohydrates, and RT-PCR using molecular markers. AP activity and immunological markers (SSEA-4, TRA-1-60, and TRA-1-81) were highly expressed in the colonies of these two lines (Fig. 1 E-J, E'-J'). Additionally, molecular markers of undifferentiated human ES cells, Nanog, Oct-4, Sox-2, and Rex-1 mRNA, were significantly expressed in CHA-hES3 and -4 (Fig. 2A).

The two ES cell types can produce embryoid bodies *in vitro*. Cells were injected into the testicles of male SCID mice to confirm teratoma formation. After 2 months, mice were sacrificed, and showed *ex vivo* tumor mass containing three embryonic germ layers, specifically, ectoderm (secretary epithelial sheath, neuron cells), mesoderm (smooth, skeletal muscle fiber, cartilage), and endoderm (islet of Langerhans) (Fig. 2B).

2. Messenger RNA Expression of BM in Human ES Cells and Survival after Vitrification/Warming, Depending on Incubation with STO Cells or STO-Conditioned Medium

To investigate the relationship between ECM expression and survival rate of ES cells after cryopreservation, BM mRNA expression before vitrification and cell morphology

Table 1. Comparison of survival and differentiation rates of sub-cultured human ES cell clumps after vitrifying/thawing according to period of extended incubation with STO feeder cells

ES cells	Incubation time	No. of vitrified/warmed ES cell-clumps	No.(%) of survived ES cell-clumps	No. (%) of spontaneously differentiated
CHA-hES-3	1 hour[1]	45	18 (40.0)[a]	6 (33.3)[a]
	16 hours[2]	55	44 (80.0)[b]	3 (6.8)[b]
CHA-hES-4	1 hour	45	20 (44.4)[a]	6 (30.0)[a]
	16 hours	42	35 (83.3)[b]	7 (5.3)[b]

[1] Sub-cultured human ES cellclumps incubated with STO for 1 h.

[2] Sub-cultured human ES cell clumps incubated with STO for 16 h.

Values with different superscripts in the column are significantly different ($P<0.05$).

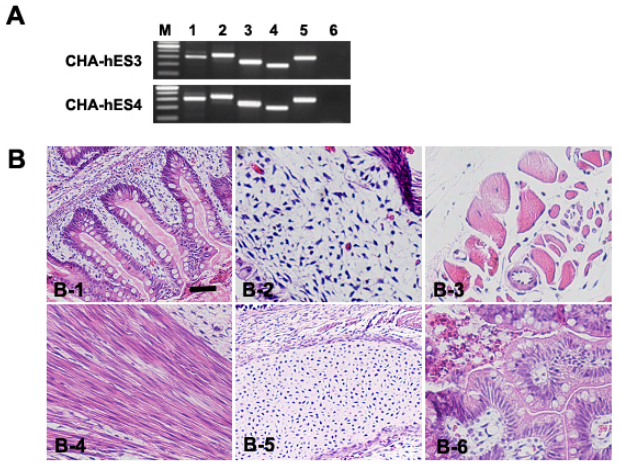

Fig. 2. (A) Expression of marker genes in human ES cell lines. M, molecular marker (100 bp ladder). Lane 1, GAPDH (310 bp). Lane 2, Nanog (340 bp). Lane 3, Oct-4 (250 bp). Lane 4, Sox-2 (220 bp). Lane 5, Rex-1 (302 bp). (B) Histology of teratomas formed by injection of hES cells (56 passages) into testicles of SCID mice. Tissues were stained with hematoxylin and eosin. B-1, secretary epithelial sheath (ectoderm). B-2, neuron cells (ectoderm). B-3, smooth muscle fiber (mesoderm). B-4, skeletal muscle fiber (mesoderm), B-5, cartilage (mesoderm). B-6, islet of langerhans (endoderm). Bar indicates 100 μm.

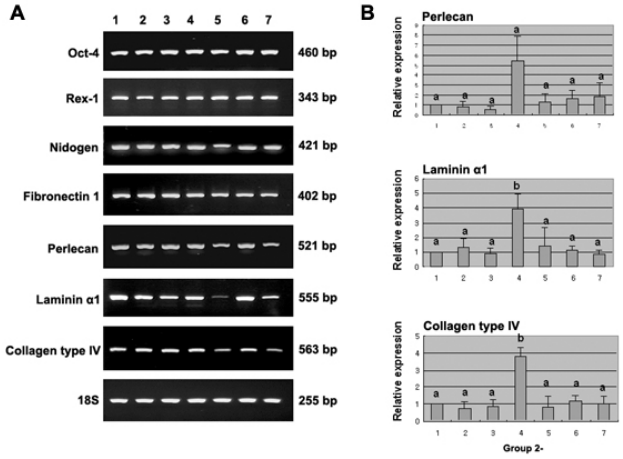

Fig. 3. Effects of extended culture of STO feeder or STO-conditioned medium (CM) on the expression of stemness- or BM-related genes. (A) Semi-quantitative analysis by RT-PCR. (B) Relative quantification by real time-RT-PCR. Lane 1, naive ES cells. Lane 2, human ES cell clumps incubated for 1 h in ES medium. Lane 3, human ES cell clumps incubated for 1 h in ES medium containing STO feeder cells. Lane 4, human ES cell clumps incubated for 16 h in ES medium containing 1-day-old STO feeder cells. Lane 5, human ES cell clumps incubated for 16 h in ES medium containing 5-day-old STO feeder cells. Lane 6, human ES cell clumps incubated for 16 h in ES medium containing 1-day-old STO-feeder cell-conditioned medium. Lane 7, human ES cell clumps incubated for 16 h in ES medium containing 5-day-old STO-feeder cell-conditioned medium. Values with different superscripts in the column are significantly different (P<0.05).

after warming were analyzed in ES cells incubated with/out feeder cells or CM. ES cells incubated with/out feeder cells or CM did not display altered mRNA expression of molecular markers (Oct-4 and Rex-1) and several ECM genes (Nidogen and Fibronectin 1). However, the transcription patterns of Perlecan, Laminin α1, and Collagen type IV were modified, as revealed by semi-quantitative RT-PCR (Fig. 3A). Accordingly, we re-analyzed the relative expression of these three genes using real-time RT-PCR. However, Laminin α1, and Collagen type IV transcripts were significantly increased only in ES cells incubated with fresh feeder for 16 h (Group 2-4) (Fig. 3B).

Survival rates of sub-clumps of CHA-hES3 cells were higher in Group 2-4 (incubated with fresh feeder cells for 16 h) than other groups, and similar expression patterns of the three BM proteins were observed (Table 2). In Group 2-6 (incubated with CM of fresh feeder for 16 h), the survival rate was slightly increased, compared with other groups. Spontaneous differentiation was decreased in all groups incubated with feeder or −CM.

DISCUSSION

Cryopreservation of human ES cells is essential if such cells are to be used in a clinical setting for regenerative medicine. An effective freezing technique would allow the preservation of early passage stocks or stable characterization of ES cells. In fact, trisomy 12 and 17 in human ES

Table 2. Comparison of survival and differentiation rates of sub-cultured CHA-hES3 clumps after vitrifying/thawing according to method of extended incubation

Incubation time[1]	Groups		No. of vitrified/warmed ES cell-clumps	No.(%) of survived ES cell-clumps	No. (%) of spontaneously differentiated
1 hour	ES-medium[1]		85	42 (49.4)[a]	16 (38.1)[a]
	STO[2]		82	43 (52.4)[a]	10 (23.3)[b]
16 hours	STO	New[3]	62	48 (77.4)[b,c]	7 (14.6)[b]
		Old[4]	64	41 (64.1)[a,b]	4 (9.8)[b]
	STO-CM	New[5]	67	41 (61.2)[a]	5 (12.2)[b]
		Old[6]	78	40 (51.2)[a]	6 (15.0)[b]

[1] human ES cell clumps incubated for 1 h in ES medium (Group 2-2).
[2] human ES cell clumps incubated for 1 h in ES medium containing STO feeder cells (Group 2-3).
[3] human ES cell clumps incubated for 16 h in ES medium containing 1-day-old STO feeder cells (Group 2-4).
[4] human ES cell clumps incubated for 16 h in ES medium containing 5-day-old STO-feeder cells (Group 2-5).
[5] human ES cell clumps incubated for 16 h in ES medium containing 1-day-old STO feeder cell-conditioned medium (Group 2-6).
[6] human ES cell clumps incubated for 16 h in ES medium containing 5-day-old STO feeder cell-conditioned medium (Group 2-7).
Values with different superscripts in the column are significantly different ($P<0.05$).

cells is occasionally observed during long-term *in vitro* culture (Ludwig et al., 2006; Draper et al., 2004). Generally, a slow cooling method using low concentrations of cryoprotective agents is effective for various types of cells or cell lines. However, these conventional methods are not applicable to primate and human ES cell lines, which either die immediately after thawing, or differentiate. Accordingly, a rapid cooling method that can avoid re-crystallization of cell contents [13] has been applied for the cryopreservation of mammalian oocytes or embryos (Rall and Fahy, 1985; Yoon et al., 2000; Choi et al., 2000). Despite several successful trials on the cryopreservation of human ES cells, survival rates are variable, compared to somatic cells or embryos (Reubinoff et al., 2001; Fujioka et al., 2004; Zhou et al., 2004).

Recently, Xu et al. (2001) reported that human ES cells on Matrigel can be successfully maintained in feeder-free culture system using MEF-CM. Soluble factors and ECM produced by feeder cells may be important for the proliferation and maintenance of undifferentiated human ES cells (Xu et al., 2001; Richards et al., 2002). Proteome analysis revealed a large number of proteins in CM from STO, including those that participate in cell growth and differentiation, ECM formation and remodeling, and also unexpectedly revealed many intracellular proteins (Lim and Bodner, 2002). Moreover, Xie et al. (2004) suggested that inactive post-radiation MEF expressed novel proteins that may underlie the molecular mechanisms to maintain pluripotency in human ES cells. In the present study, co-culture with feeder cells induced tight aggregation of human ES sub-clumps (Fig. 3B, C) and increased the survival rate after vitrifying/warming (Table 1). Occasionally, sub-clumps of human ES cells were tightly attached to the bottom of feeder cells after 16 h of incubation, and could not be detached by gentle pipetting. However, most sub-clumps were detached from the bottom of culture dishes as a highly compact cell mass (Fig. 1B, C). Transcript levels of molecular markers and BM components were additionally analyzed in human ES sub-clumps incubated with/out STO feeder cells. Messenger RNA expression of stemness markers of ES cells and some BM components (Nidogen and fibronectin 1) were not altered in all groups. Overall, 3 BM components (Perlecan, Laminin α1, and Collagen type IV) displayed increased mRNA expression in co-cultured groups (Fig. 3A). Accordingly, we analyzed their relative expression patterns using real-time RT-PCR. Relative

mRNA expression was increased when human ES cell sub-clumps were incubated with fresh feeder cells for 16 h (Fig. 3B). Moreover, the survival rate of ES cells in this group after vitrifying/warming was higher than those of other groups (Table 2). CM of STO feeder cells did not fully support BM expression of ES cells or survival after vitrifying/warming. However, we observed partly tight aggregation and increased survival rate, which was not significant. Therefore, the effects of FPF on the regulation of other ECMs or mechanical interaction between cells (ES cell-ES cell or ES cell-feeder cell) should be examined to elucidate the mechanisms for maintaining human ES cells. Our findings indicate that incubation of human ES cells with fresh feeder cells before vitrification increases ECM expression and protects against cryoinjury by retention of cell-to-cell interactions during cryopreservation.

Two (CHA-hES3 and -4) were used in our experiments. High survival rates of more than 80% and low spontaneous differentiation in extended incubation groups with STO feeder cells were similar between the two cell lines after vitrifying/warming (Table 1). Therefore, this vitrification method after incubation with feeder cells is a useful tool for other human ES cell lines. Both CHA-hES3 and -4 lines have been effectively maintained over 60 passages to date (Fig. 1D), and display normal characteristics of human ES cells (Fig. 1E-J).

Cryopreservation of human ES cells has important implications for clinical application in regenerative medicine. However, despite intensive research using conventional slow cooling or vitrification, efficiency is still variable. In the present study, we have developed a simple and efficient cryopreservation system for human ES cells showing a high survival rate of more than 80% and low differentiation, using vitrification and simple extended incubation with feeder cells. Extended incubation with feeder cells may increase the expression of BM proteins in human ES cells and cause strong aggregation between cells, resulting in high survival and low spontaneous differentiation rates after cryopreservation. However, the precise mechanisms for maintaining stemness by soluble factor(s) from feeder cells or biophysical properties require further investigation.

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