

A simple and efficient cryopreservation method for primate embryonic stem cells

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ABSTRACT Human embryonic stem (ES) cells have the potential to differentiate into all cell types. As these cells may be able to provide an unlimited cell source for transplantation therapies, it is necessary to establish reliable methods for their handling and manipulation, including human ES cell cryopreservation. Here, we report the development of a simple and efficient cryopreservation method for primate ES cell lines using vitrification in conventional cryovials. Using standard slow-rate cooling methods, the cryopreservation efficiency for cynomolgus monkey ES cell lines was approximately 0.4%, while that for a human ES cell line was virtually 0%. Primate ES cell lines, however, were successfully cryopreserved by the present vitrification method using conventional cryovials yielding a survival rate of about 6.5% for monkey ES cells and 12.2% for human ES cells. Vitrified ES cells quickly recovered after thawing and exhibited a morphology indistinguishable from non-vitrified cells. In addition, they retained a normal karyotype and continued to express ES cell markers after thawing. Thus, our vitrification ES cell cryopreservation method expands the utility of primate ES cells for various research and clinical purposes.

KEY WORDS: *primate embryonic stem cell, human embryonic stem cell, cryopreservation, vitrification*

Introduction

Embryonic stem (ES) cell lines are derived from the inner cell mass of blastocysts and can proliferate indefinitely *in vitro*. The defining feature of these cells is their potency to differentiate into a variety of cell types of all three embryonic germ layers: the ectoderm, mesoderm and endoderm (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000). Thus these characteristics make ES cell lines valuable for research in developmental biology and transplantation therapy (Keller and Snodgrass, 1999). Non-human primate ES cells, such as cynomolgus monkey ES cells, have been extensively used as a model system in preclinical experiments to examine the clinical applications of human ES cells (Thomson *et al.*, 1995 and 1996; Suemori *et al.*, 2001). The sensitivity of both human and monkey ES cells to cryopreservation, however, has severely limited the utility of these cells (Reubinoff *et al.*, 2001). For example, difficulties in the distribution of cell lines have made the reproduction of experiments using primate ES cells problematic. Therefore, it is necessary to establish reliable methods for the cryopreservation of primate ES cells. Slow-rate cooling methods using dimethylsulfoxide (DMSO) as a cryoprotectant are effective

for a wide variety of cell lines, including mouse ES cell lines. In most protocols, cells are suspended in freezing medium containing DMSO at 5-20%, transferred into glass or plastic cryovials and then frozen by cooling at 1.0 to 2.0°C/min. The ability to freeze the majority of cell lines with common laboratory equipment to yield apparatus with satisfactory survival rates after thawing has made these protocols widely used. Such conventional methods, however, are not applicable to primate ES cell lines, as these cells die immediately after thawing. Although the reason why primate ES cells cannot survive the freeze-thaw process is not clear, damage by ice crystal formation in the cytoplasm during the freezing process is one of the possible causes of cell death (Dobrinisky, 1996). A rapid cooling method, vitrification, has been used for the cryopreservation of fertilized eggs or embryos of several mammalian species, including human (Rall and Fahy, 1985; Kuleshova *et*

Abbreviations used in this paper: DAP medium, DMSO+Acetamide+Propylene glycol; DES medium, DMSO+Ethylene glycol+Sucrose; DMSO, dimethylsulfoxide; EFS medium, Ethylene glycol+Ficoll+Sucrose; ES, embryonic stem; MEF, mouse embryonic fibroblast; OPS, opened pulled straws.

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al., 1999; Dobrinsky, 2002), to prevent ice crystal formation (Luiet, 1937). Therefore, we postulated that primate ES cells may be effectively cryopreserved by vitrification. Although a report demonstrating that human ES cells could be successfully cryopreserved by vitrification (Reubinoff *et al.*, 2001) supports this hypothesis, the protocol was both labor consuming and impractical on a large scale, as it requires a special device of open pulled straws (OPS) and the manual picking-up of individual stem cell colonies. In addition, this technique encourages potential contamination with pathogenic agents (Gorman and Patterson, 1995) due to the direct exposure of ES cell suspensions to liquid nitrogen filled in OPS. Such risks must be avoided for the potential clinical use of human ES cells. Therefore, we developed a novel cryopreservation method for primate ES cells that is simple and efficient on a large scale.

Experimental Procedures

ES cell culture

The cynomolgus monkey ES cell lines CMK6 and CMK9 were established as described (Suemori *et al.*, 2001) and cultured on mitomycin C-treated mouse embryonic fibroblast (MEF) feeder cell layers. Monkey ES were cultured in CMK medium consisting of DMEM/F-12 supplemented with 0.1 mM 2-mercaptoethanol, 20% KSR (Invitrogen Corp., CA, USA), 2 mM L-glutamine and 1% MEM nonessential amino acids. The establishment and culture of human ES cell line, KhES-1, are described elsewhere (H. Suemori, in preparation). Briefly, human ES cells were cultured in CMK medium supplemented with 5 ng/ml recombinant human FGF-2 (Upstate, VA, USA) on MEF feeder cells and the ES cells were subcultured every 3-4 days by enzymatic dissociation using a dissociation solution consisting of 0.25% trypsin, 1 mg/ml collagenase, 1 mM CaCl₂ and 20% KSR in PBS. The establishment and use of human ES cell lines was performed in conformity with «The Guidelines for Derivation and Utilization of Human Embryonic Stem Cells (2001)» of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

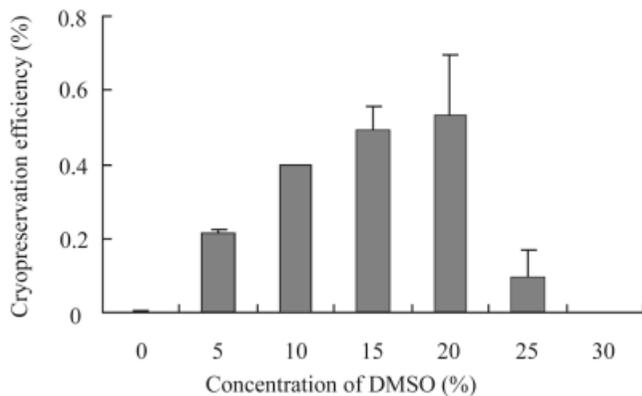


Fig. 1. Cryopreservation efficiency of CMK6 ES cells by slow cooling method. CMK6 ES cells were frozen in freezing media containing 0 to 30% DMSO. The number of ES cell colonies was counted at 6 days after thawing and compared to the control plate.

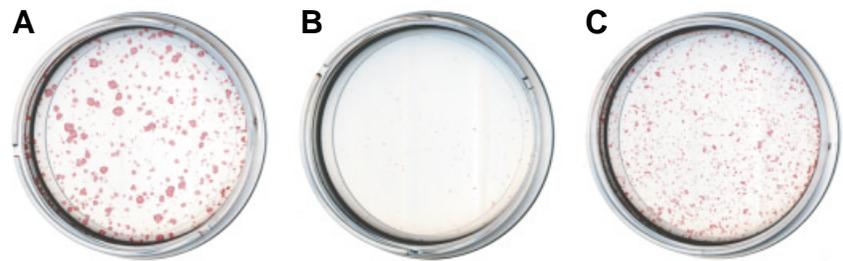


Fig. 2. Typical morphology of CMK6 ES cell colonies after subculturing, slow-cooling and vitrification. (A) A control plate prepared by passaging as described in Experimental Procedures. ES cell colonies at five days after cryopreservation by (B) the slow-cooling and (C) the vitrification method.

Slow-rate cooling method

Confluent cultures of ES cells in 100 mm plates were dissociated using in dissociation solution. ES cell suspensions were divided into three centrifugation tubes containing equal cell numbers and one tenth of each suspension was replated onto feeder layers, cultured and used as a control for calculating cell viability after thawing. After centrifugation, the cell pellets were resuspended in freezing medium containing 10% DMSO in CMK medium and transferred to 1 ml cryovials (Nalge Nunc International, NY, USA, SYSTEM 100 Cryogenic Vial). The cryovials were placed into a Nalgene™ Cryo1°C Freezing Container (Nalge Nunc International). ES cells were stored in a -80°C freezer overnight, then transferred to a -150°C freezer until thawing.

The ES cells were thawed at 37°C in a water bath with gentle shaking and the ES cell suspension was transferred into a centrifugation tube filled with 10 ml CMK medium. After centrifugation, the supernatant was removed and the ES cell pellets were resuspended in 2 ml CMK medium. The cells were then plated in 6 well culture plates or 35 mm culture dishes with a feeder layer, cultured for 6 days and then evaluated for their survival rates.

Vitrification methods

ES cells were prepared as described above. Three kinds of freezing media, DAP, DES and EFS40, were examined in our simple vitrification method. The composition of each freezing medium was as follows. DAP: 2 M DMSO, 1 M acetamide and 3 M propylene glycol in CMK medium; DES: 20% DMSO, 20% ethylene glycol and 0.5 M sucrose in CMK medium and EFS40: 40% ethylene glycol, 18% ficoll 70,000 MW and 0.3 M sucrose in CMK medium. After resuspending the ES cells in 200 µl of freezing medium, samples were transferred to cryovials and immediately soaked in liquid nitrogen so that 2/3 of the vial from the bottom up was immersed, thereby avoiding contamination by the coolant. The cryovials were then stored in a -150°C freezer until thawing.

To thaw the vitrified ES cells, medium pre-warmed to 37°C was added to the vials. The cells were thawed quickly by pipetting and after centrifugation, ES cells were plated as described for the cells subjected to slow cooling as described above. The thawed cells were cultured for 4 days and evaluated for their survival rates.

Assessment of ES cell growth after cryopreservation

To evaluate the survival rate after cryopreservation, the efficiency of the slow-rate cooling method was assessed by counting the number of ES cell colonies, while that of the vitrification method

was assessed by counting ES cell numbers. The number of ES cells or ES cell colonies was counted and compared to that of the control plates prepared as described above.

Histochemical analysis of recultured ES cells

ES cells were fixed in 4% paraformaldehyde for 10 min. Alkaline phosphatase activity was detected with Vector Red Alkaline Phosphatase Substrate Kit I (Vector laboratories, CA, USA). The expression of SSEA-4 and TRA1-60 was detected by immunostaining using specific antibodies (CHEMICON International Inc., CA, USA) diluted at 1:100. After 2 hours of incubation, antibody staining was visualized with an HRP-labeled secondary antibody and diaminobenzidine.

Teratoma formation

Several passages after thawing, ES cells were dissociated. Approximately 1×10^7 ES cells were subcutaneously injected into SCID mice. Teratomas were formed within 5 to 8 weeks after injection. Teratomas were removed by dissection, fixed in 4% formalin, embedded in paraffin, sectioned at 5 μ m and stained with hematoxylin and eosin.

Results and Discussion

Slow-rate freezing of monkey ES cells

For biological research and medical applications, it is necessary to develop an efficient cryopreservation method for the primate ES cell lines, CMK6 and CMK9. We therefore have attempted to establish an efficient and simple cryopreservation protocol applicable to primate ES cells. We first examined the efficiency of the slow-rate cooling method using freezing medium containing 10% DMSO for primate ES cells. It was necessary to determine the cell number after thawing to accurately evaluate the survival rates. However, this was impossible because of extremely poor recovery associated with this method (Fig. 2B). Thus, we estimate the survival rates by counting the number of colonies. After thawing, very few CMK6 cells remained alive, resulting in a cryopreservation efficiency of 0.4% compared to that for passaging (Fig. 1). To

optimize the cryopreservation medium, we tested several concentrations of DMSO ranging from 0-30%. Although 20% DMSO proved to be most effective for slow cooling, the efficiency was only increased to 0.6%. We also examined a range of commercially available freezing media and tested the effect of additional components such as methylcellulose (Ohno *et al.*, 1988) on the freezing efficiency. However the cell viability after thawing scarcely improved.

From these results, primate ES cells were found to exhibit low survival rates after cryopreservation using the slow-rate cooling method. Two weeks or more of expansion was required for undifferentiated stem cells to proliferate enough for subculturing. Thus, this time consuming and unreliable protocol is not practical for laboratory and clinical applications of primate ES cells.

Vitrification freezing of monkey ES cells

Vitrification is an alternative way to cryopreserve a variety of cell types, including mammalian fertilized eggs or embryos (Rall and Fahy, 1985; Kuleshova *et al.*, 1999; Dobrinsky, 2002). Although vitrification of human ES cells has been previously attempted (Reubinoff *et al.*, 2001), the potential contamination risks (Gorman and Patterson, 1995) and limited utility of this method has made it necessary to develop more efficient, reliable and simple vitrification methods.

As cryovials are the most commonly used cryopreservation containers for cultured cells, we replaced the OPSS used in this previously described method to include these vials. We also compared the efficacy of three freezing media, DES, DAP and EFS40, to optimize the vitrification protocol using cryovials. While DES (DMSO, Ethylene glycol and Sucrose) was used for the vitrification of human ES cells using OPS (Reubinoff *et al.*, 2001), DAP (DMSO, Acetamide and Propylene glycol) is used for the cryopreservation of mammalian oocytes and embryos (Nakagata *et al.*, 1993). EFS (Ethylene glycol, Ficoll and Sucrose) is also used for the cryopreservation of mammalian embryos in glass capillaries (Kasai *et al.*, 1990; Nagy *et al.*, 2003). The concentration of ethylene glycol in EFS medium varies widely among

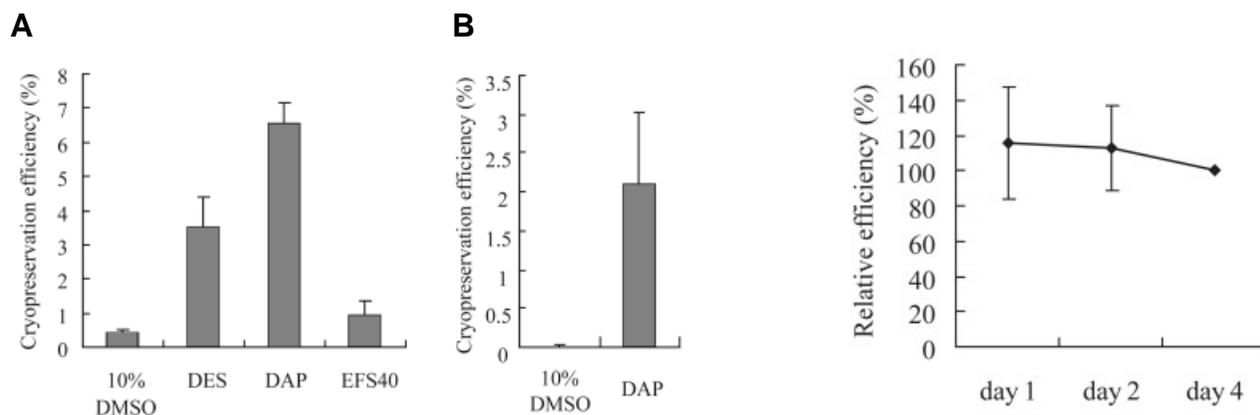


Fig. 3 (Left). Efficiency of cryopreservation by vitrification. (A) The average survival rate of CMK6 ES cells after vitrification using DES, DAP and EFS40 freezing media was estimated by counting cell numbers at four days after thawing. The efficiency of the slow-cooling method using 10% DMSO is also shown. (B) Cryopreservation efficiency for CMK9 cell lines was examined as in (A).

Fig. 4 (Right). Recovery of ES cells after cryopreservation. The survival rate of CMK6 ES cells was evaluated 1, 2 and 4 days after thawing from vitrification using DAP. The cryopreservation efficiencies for day 1 and day 2 relative to day 4 are shown.

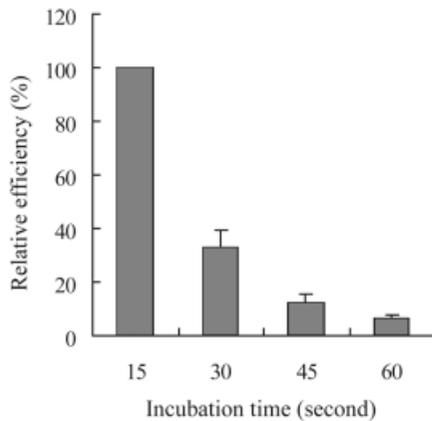


Fig. 5. Effect of incubation time in DAP during vitrification on the cryopreservation efficiency of CMK6 ES cells. ES cells were incubated in DAP for 15, 30, 45 and 60 seconds before freezing. The cryopreservation efficiency was then evaluated 4 days after thawing and it was found that the efficiency rapidly decreased in accordance with the increase in incubation time.

protocols. We chose the EFS40 medium containing 40% v/v ethylene glycol, as this medium is widely used for mouse embryo cryopreservation. Since the vitrification methods yielded higher cell recovery rates than did the slow-rate freezing methods as shown in Fig. 2, we were able to accurately evaluate the survival rates by determining the cell number instead of the colony number. Among the vitrification freezing media examined, DAP exhibited the highest recovery rate of 6.5% (Fig. 3A), nearly twice

as high as the efficiency using DES (3.5%). EFS40 displayed a very low efficiency of 1.0%.

The cryopreservation efficiency was also examined using another cynomolgus monkey ES cell line, CMK9. The viability of vitrified CMK9 cells was about 2.1% (Fig. 3B). It was very low compared with that of CMK6 cells, which was about 6.5%. Such differences may represent a higher sensitivity of CMK9 cells to mechanical damage as suggested by previous experiments (Furuya *et al.*, 2003).

We next tried to optimize this vitrification method using DAP. We examined a range of DAP concentrations from 0.25-1.5 fold of the initially tested concentration. The original concentration of DAP proved to be most effective. We attempted to improve the freezing efficiency by varying the ratios of the DAP components. Although we tested concentrations of DMSO ranging from 15-30% and propylene glycol ranging from 15-30%, we found little improvement in efficiency compared to the original DAP medium (data not shown).

We further analyzed the cryopreservation efficiency for CMK6 ES cells by vitrification using DAP immediately after thawing. As shown in Fig. 4, the cells exhibited rapid recovery from cryopreservation and exhibited similar survival rates at 1, 2 and 4 days after thawing.

In a subset of previously reported vitrification protocols, pretreatment with a less hypertonic medium than the freezing medium improved the efficiency of cryopreservation (Rall and Fahy, 1985). For the OPS vitrification method, ES cells were exposed to 1 M DMSO for 1 min before freezing. Therefore, we pretreated CMK6 cells with 1 M DMSO for 1 min before vitrification in DAP. This pretreatment, however, did not significantly alter the cryopreservation efficiency.

As vitrification freezing media contain high concentrations of cryoprotectants, these media are toxic to ES cells. Therefore, we examined the effect of the duration of time the cells spent suspended in the freezing medium before soaking in liquid nitrogen on cell survival after thawing. The survival rates decreased with increased holding time before freezing (Fig. 5); when the holding time was longer than 1 min, cryopreservation efficiency was lower than 0.5%. Therefore, EFS40 did not permit good recovery rates (Fig. 3A), probably because it is too viscous to handle rapidly. A

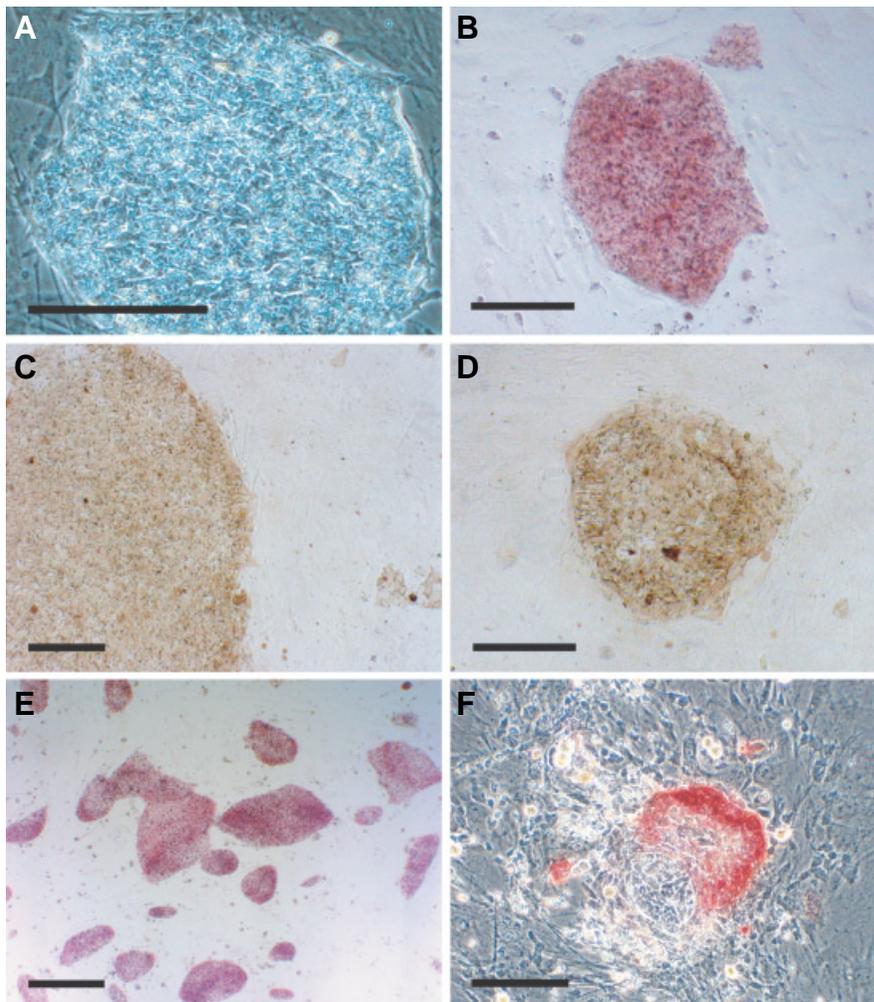


Fig. 6. Morphology and stem cell marker expression in CMK6 ES cells after cryopreservation. A phase contrast microscopic image of vitrified CMK6 at 3 days after thawing (A) and vitrified CMK6 cells examined for alkaline phosphatase activity (B, E-F). Immunocytochemistry of vitrified CMK6 detected the expression of SSEA-4 (C) and TRA1-60 (D). A low power view of vitrified ES cells at 5 days stained for alkaline phosphatase activity showing that most colonies retained their undifferentiated state (E). A typical differentiating colony (F). Scale bars, 20 μ m (A-D, F); 50 μ m (E).

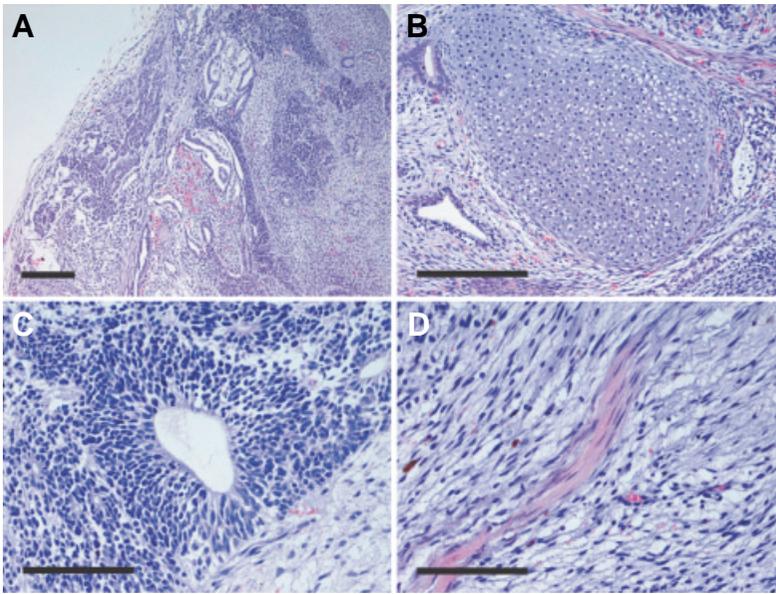


Fig. 7. Teratoma formation by vitrified CMK6 ES cells. The gross appearance (A) and higher magnification views (B-D) of a H & E-stained section of a teratoma produced from vitrified CMK6 ES cells. Cells expressing cartilage (B), neuroepithelium (C) and muscle (D) phenotypes were identified. Scale bars, 20 μm (A, B); 10 μm (C, D).

holding time of approximately 15 s is thought to be the minimal handling time required for suspending cells, transferring to vials and freezing. Then, the greater time needed to manipulate the cells until freezing leads to increased cell death due to cryoprotectant toxicity.

We also examined the effect of the cell concentration in the cryovials on the survival rate. CMK6 cells were subjected to our vitrification protocol over a wide range of cell concentrations, from 5×10^5 to 1×10^7 cells/vial. Regardless of the cell density, ES cells were successfully recovered at similar efficiencies. This result suggested that primate ES cells can be cryopreserved in cryovials for any culture scale, from 35 mm dishes to 100 mm or larger. The independence of the efficiency from culture size makes this protocol advantageous for a variety of clinical and research applications using primate ES cells.

Reubinoff *et al.* demonstrated that 100% of the human ES cell colonies could be recovered after cryopreservation using the OPS vitrification method (Reubinoff *et al.*, 2001). This difference in efficiency from our protocol was likely caused by differences in the vitrification protocols and assay systems. They evaluated the recovery efficiency by comparing colony numbers before and after vitrification, but the actual cell survival rate was not presented. In addition, more than 70% of the colonies exhibited a differentiated phenotype at 7 days after thawing by their OPS vitrification method. Using our culture system, spontaneous differentiation of primate ES cells was rarely observed (Fig. 6 E,F); less than 1% of the colonies exhibited differentiated morphology during routine maintenance. The rate of occurrence of differentiated colonies increased to 3% after vitrification and this might have been caused by a relatively higher tolerance of differentiated cells to damage induced by the vitrification processes. Thus, our vitrification method induces differentiation at a lower rate.

Cell characteristics after cryopreservation

Our vitrification method using DAP freezing medium permitted the rapid recovery of ES cells after cryopreservation. Usually, approximately two weeks are required following cryopreservation by slow-rate cooling for human ES cells to grow to the cell number present at the time of freezing. By our vitrification method, this number of cynomolgus monkey ES cells was recovered within about 4 days. The thawed ES cells tend to form small colonies compared with control ES cells probably because of additional dissociation caused by handling during freezing/thawing and damage induced by the preservation process (Fig. 2). Vitrified ES cells recovered very quickly and could be maintained for more than one month without any signs of differentiation or changes in their growth properties. Since human ES cells tend to differentiate after thawing (Reubinoff *et al.*, 2001), our vitrification method may be an effective way to minimize this side effect. Such quick and safe recovery of ES cells also reduces the time and cost of experiments requiring cryopreservation from those using conventional slow-rate cooling methods.

Following the culture of vitrified ES cells, we assessed their stem cell marker expression (Suemori *et al.*, 2001). Thawed cynomolgus monkey ES cells continued to exhibit the alkaline phosphatase activity characteristic of ES cells and also expressed the stem cell markers SSEA-4 and TRA1-60 (Fig. 6). We also analyzed the karyotype of CMK6 cells post-vitrification, demonstrating that these ES cells retained a normal karyotype of 40+ XY chromosomes (data not shown). To examine differentiation potential of vitrified ES cells, we examined the formation of teratomas by injecting ES cells after vitrification into SCID mice. Teratoma formation was observed in two independent experiments. Histological analysis of isolated teratomas revealed that the vitrified ES cells differentiated into a variety of cell types, including cartilage, neuroepithelium and muscle (Fig. 7). Thus, the vitrified ES cells retained their pluripotency.

Human ES cell cryopreservation

We examined the potential application of this vitrification method to cryopreservation of a human ES cell line, KhES-1. We could not recover any viable cells after cryopreservation by the slow-rate cooling method using 10% DMSO (Fig. 8). In contrast, human ES cells both survived and continued to grow after vitrification in DAP. The cryopreservation efficiency of KhES-1 was approximately 12.2%. As seen for monkey ES cells after vitrification, the vitrified human ES cells were indistinguishable from non-vitrified ES cells.

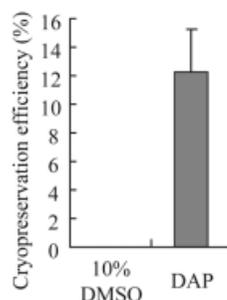


Fig. 8. Cryopreservation efficiency of a human ES cell line. The survival rates of a human ES cell line, KhES-1, after cryopreservation by the slow-cooling method using 10% DMSO and by the vitrification method using DAP.

In conclusion, we have developed a novel cryopreservation method for primate ES cell lines, including human ES cell lines. This vitrification method provides the simple, efficient and reliable cryopreservation of primate ES cell lines. Such a system will be exceedingly helpful for experimental research and medical applications.

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