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Effective cryopreservation of human embryonic stem cells by the open pulled straw vitrification method

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BACKGROUND: Human embryonic stem (ES) cells originate from the inner cell mass of the blastocyst, and retain in culture the properties of pluripotent cells of the early embryo. The study aim was to determine whether the open pulled straw (OPS) vitrification method, which is highly effective for the cryopreservation of embryos, might be also efficient for human ES cells. METHODS AND RESULTS: All human ES cell clumps that were vitrified by the OPS method could be recovered upon thawing, and gave rise to ES cell colonies after plating. Vitrified colonies were significantly smaller and showed an increased level of background differentiation compared with control colonies. However, these unwanted effects could be overcome by additional cultivation of the colonies for 1 and 2 days respectively. The vitrified human ES cells were cultivated for prolonged periods and retained the properties of pluripotent cells, including a normal karyotype, expression of the transcription factor Oct-4 and surface markers that are characteristic to human ES cells. When grafted into SCID mice, the vitrified cells gave rise to teratomas containing derivatives of all three embryonic germ layers. CONCLUSIONS: Vitrification by the OPS method is reliable and effective for the cryopreservation of human pluripotent embryonic stem cells.

Key words: cryopreservation/human embryonic stem cells/vitrification

Introduction

Embryonic stem (ES) cell lines are derived from the pluripotent cells of the inner cell mass of the blastocyst. These cell lines can be propagated indefinitely *in vitro*, yet still retain a normal karyotype and the capacity for differentiation into a wide variety of somatic tissues representing progeny of the three embryonic germ layers. ES cell lines have been derived recently from human blastocysts (Thomson *et al*., 1998; Reubinoff *et al*., 2000) and are expected to have far-reaching applications in the areas of regenerative medicine, pharmacology and basic scientific research. Human ES cells provide a powerful in-vitro model for the study of early human embryology. They may be used for the development of new drugs and for the discovery of new growth and differentiating factors that may have therapeutic applications. Given the self-renewal potential of ES cells, combined with their capability to differentiate into any cell type, these cells can potentially provide an unlimited source of cells for transplantation therapy (Keller and Snodgrass, 1999).

In order to exploit this remarkable potential of human ES cells, improvement of currently used technologies for handling and manipulating the cells is required. In this context, an efficient cryopreservation method would be highly valuable for the development and widespread use of these cell lines. Effective freezing and thawing techniques would enable the efficient preservation of stocks of early passage cells, as well as the conservation of specific clones that are developed from the original cell lines such as genetically modified clones. Moreover, efficient cryopreservation will be essential if human ES cell banks are to be established (Gearhart, 1998). Lastly, effective freezing and thawing technologies will allow effortless transfer of the cells between research centres, promoting scientific collaboration and facilitating widespread use of the cells for research and clinical applications.

Slow-rate freezing and rapid thawing methods are most commonly used for the cryopreservation of cell lines (Freshney, 1994). While these standard methods are efficient for the cryopreservation of mouse ES cells (Robertson, 1987), it has been observed that the survival of undifferentiated human ES cells following slow-rate freezing is very poor, and most of the cells either differentiate or die.

As embryonic stem cells originate from the pluripotent cells of the blastocyst and retain in culture the properties of these cells such as morphology, gene expression and pluripotentiality, it has been postulated that methods developed specifically for the cryopreservation of blastocysts might be also efficient with human ES cells.

Vitrification has been extensively studied as a method of cryopreservation of embryos, and was recently shown to be highly efficient in bovine species (Vajta *et al*., 1997a, 1998a), as well as in the pig and hamster (Vajta, 1997b; Lane *et al*., 1999a), all of which poorly withstand freezing and thawing by other methods. Moreover, initial data suggest that vitrification methods may be also beneficial for the cryopreservation of human blastocysts (Lane *et al*., 1999b; Yokota *et al*., 2000). Here, the use of vitrification for the cryopreservation of human ES cells is reported for the first time.

Materials and methods

Human ES cell culture

Human ES cell lines HES-1 and HES-2 were used to evaluate the efficiency of vitrification for the cryopreservation of human ES cells. The derivation and characterization of these cell lines have been described previously (Reubinoff *et al*., 2000). Human ES cell colonies were cultured on a mouse embryonic fibroblast feeder layer in gelatin-coated tissue culture dishes. The culture medium consisted of Dulbecco's modified eagle medium (DMEM) (Gibco, Gaithersburg, MD, USA) without sodium pyruvate but containing glucose (4500 mg/l) and supplemented with 20% fetal bovine serum (FBS: Hyclone, Logan, UT, USA), 0.1 mmol/l β-mercaptoethanol, 1% non-essential amino acids, 2 mmol/l glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin (Gibco). The colonies were propagated in clumps of about 100 undifferentiated cells, on a mouse feeder layer, about every 7 days. The clumps were dissociated with a combined approach of mechanical slicing followed by exposure to dispase (10 mg/ml in serum-containing medium; Gibco).

ES cell cryopreservation

ES cells were cryopreserved in clumps of about 100–200 cells by using either the conventional slow-freezing method (Freshney, 1994) or the open pulled straw (OPS) vitrification method (Vajta *et al*., 1998a). The clumps were harvested as described above for routine passage.

With the slow conventional method, about 10–20 clumps of ES cells were transferred into a 1.2 ml cryo-vial (Nalge Nunc, Napervville, IL, USA) containing 0.5–1 ml of pre-cooled (4°C) freezing medium [90% serum and 10% dimethylsulphoxide (DMSO; Sigma Chemical Co., St Louis, MO, USA)]. The vials were slowly cooled (~1°C/min) in a freezing container (Nalgene; Nalge Nunc) to –80°C and then plunged into and stored in liquid nitrogen. The vials were rapidly thawed in a water bath at 37°C. The freezing medium was gradually diluted with 1 ml of HEPES-buffered ES cell culture medium. Thawed ES cell clumps were then washed in HEPES-buffered ES cell culture medium and plated onto a fresh feeder layer.

Alternatively, ES cell clumps were vitrified by using the OPS vitrification method (Vajta *et al*., 1988a), albeit with some modifications. French mini-straws (250 μl; IMV, L'Aigle, France) were heat-softened over a hot plate, and pulled manually until the inner diameter was reduced to about one-half of the original diameter. The straws were allowed to cool to room temperature and then cut at the narrowest point with a razor blade. The straws were sterilized by gamma irradiation (15–25 KGy). Two vitrification solutions (VS) were used. Both were based on a holding medium (HM) which included DMEM containing HEPES buffer (Gibco) supplemented with 20% FBS. The first VS (VS1) included 10% DMSO and 10% ethylene glycol (EG; Sigma Chemical Co.). The second vitrification solution (VS2) included 20% DMSO, 20% EG and 0.5 mol/l sucrose.

Table I. Characteristics of human embryonic stem (ES) cell colonies that were recovered from vitrification, in comparison with non-vitrified control colonies

	Control colonies Day 2 (n = 26)	Vitrified colonies Day 2 (n = 25)	P^*	Control colonies Day 7 (n = 26)	Vitrified colonies Day 7 (n = 25)	P^+	Vitrified colonies Day 8 (n = 25)	P^+	Vitrified colonies Day 9 (n = 25)	P^+
Area of colonies (mm^2)[a]	0.59 ± 0.14	0.28 ± 0.08	< 0.001	4.42 ± 0.78	2.63 ± 0.82	< 0.001	4.1 ± 1.31	0.3	5.6±1.7	< 0.004
No. of colonies										
Mainly undifferentiated	–	–	–	12(46)	6(24)	< 0.003	8(32)	0.048	8(32)	0.082
Mainly differentiated	–	–	–	14(54)	13(52)	–	13(52)	–	14(56)	–
Completely differentiated	–	–	–	0(0)	6(24)	–	4(16)	–	3(12)	–

Values are mean ± SD.
*Student's t-test comparison between control and vitrified colonies at day 2.
+Student's t-test and χ^2 test, comparisons between control colonies at day 7 and colonies from vitrified cells at days 7, 8 and 9.
Values in parentheses are percentages.

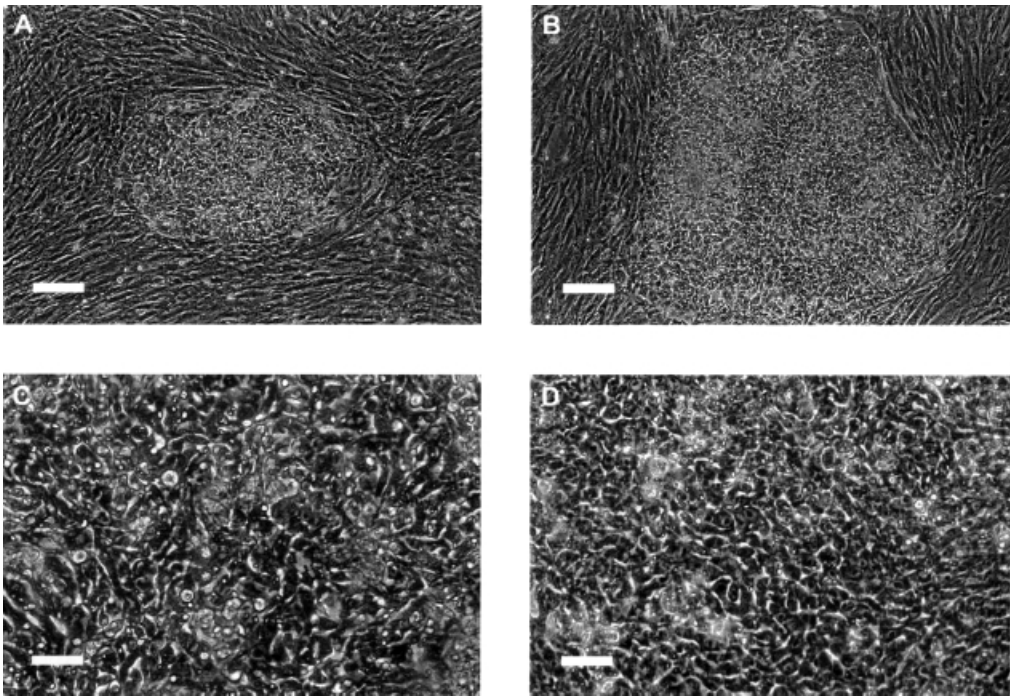

Figure 1. Phase-contrast micrographs of human embryonic stem (ES) cells after vitrification. (**A**) Colony of vitrified ES cells 2 days after plating. (**B**) Colony of control non-vitrified ES cells. (**C**) Higher magnification of an area of a vitrified ES cell colony. (**D**) An area of a control non-vitrified ES cell colony. Scale bars: A, B = 150 μm; C, D = 30 μm.

All procedures were performed on a heating stage at 37°C. Four to six clumps of ES cells were first incubated in VS1 for 1 min, followed by incubation in VS2 for 25 s. They were then washed in a 20 μl droplet of VS2 and placed within a droplet of 1–2 μl of VS2. The clumps were loaded into the narrow end of the straw from the droplet, by capillary action. The narrow end was immediately submerged into liquid nitrogen. Straws were stored in liquid nitrogen.

Thawing was also performed on a heated stage at 37°C as described previously (Vajta *et al*., 1998a), with slight modifications. At 3 s after removal from liquid nitrogen, the narrow end of the straw was submerged into HM supplemented with 0.2 mol/l sucrose. When the drop of medium at the tip of the straw was liquefied, and medium had started to fill up the straw, the wide opening of the straw was sealed. The expansion of cold air in the straw due to the gradual rise of its temperature led to expulsion of the clumps into the dish. After 1 min of incubation, the clumps were transferred and further incubated for 5 min in HM with 0.1 mol/l sucrose. The clumps were further incubated twice (5 min each incubation) in HM before being plated onto a fresh feeder layer.

Assessment of ES cell growth and differentiation after vitrification

The growth and the level of background spontaneous differentiation were compared between vitrified and non-vitrified ES cells. At the time of routine passage, clumps of undifferentiated human ES cells that originated from the same culture dish were allocated randomly either to vitrification or transferred to fresh feeder layer to serve as controls. The vitrified clumps were recovered after 2 h and plated onto the same feeder layer under the same culture conditions as the controls. The area and level of differentiation of each of the colonies that had developed from the vitrified and control clumps were evaluated 2 and 7 days after plating by a single investigator (B.R.) who was unaware of the origin of the colonies. The colonies that developed from vitrified cells were also evaluated for these parameters on the 8th and 9th days after plating. Longitudinal and horizontal diameters were measured for each colony by using a stereomicroscope. The approximate area of each colony was calculated by using the surface area equation of an ellipse ($\pi ab/4$, where a and b are the horizontal and longitudinal diameters). The level of differentiation was determined (using phase-contrast microscopy) for each colony according to its morphological appearance. Areas of tight, small cells with high nuclear:cytoplasm ratios were scored as undifferentiated, while areas with large cells with abundant cytoplasm were scored as differentiated. Each colony was graded as predominantly (>50%) undifferentiated, predominantly (>50%) differentiated, or completely differentiated. The intra- and inter-observer variabilities of these measurements were <5%.

The mean area of the colonies and the distribution of the colonies into the various levels of differentiation were compared between the study and control groups. Student's *t*-test and a χ^2 test were used for the statistical analyses, as appropriate.

Characterization of vitrified ES cells

Colonies that had originated from vitrified human ES cells were fixed in the culture dishes (at passage level 3 after vitrification for HES-1 and 17 for HES-2) using 100% ethanol for immunofluorescence demonstration of the stem cell surface markers GCTM-2, and TRA 1-60, while 90% acetone fixation was used for SSEA-4. The sources of the monoclonal antibodies used for the detection of the markers were as follows: GCTM-2, this laboratory; TRA 1-60, a gift of Peter Andrews, University of Sheffield and SSEA-4 (MC-813-70), Developmental Studies Hybridoma Bank, Iowa, IA, USA. Antibody localization was performed by using rabbit anti-mouse immunoglobulins conjugated to fluorescein isothiocyanate (Dako, Carpinteria, CA, USA).

Alkaline phosphatase activity was demonstrated (at passage level 19 after vitrification for HES-1 and 17 for HES-2) as described previously (Buehr and McLaren 1993).

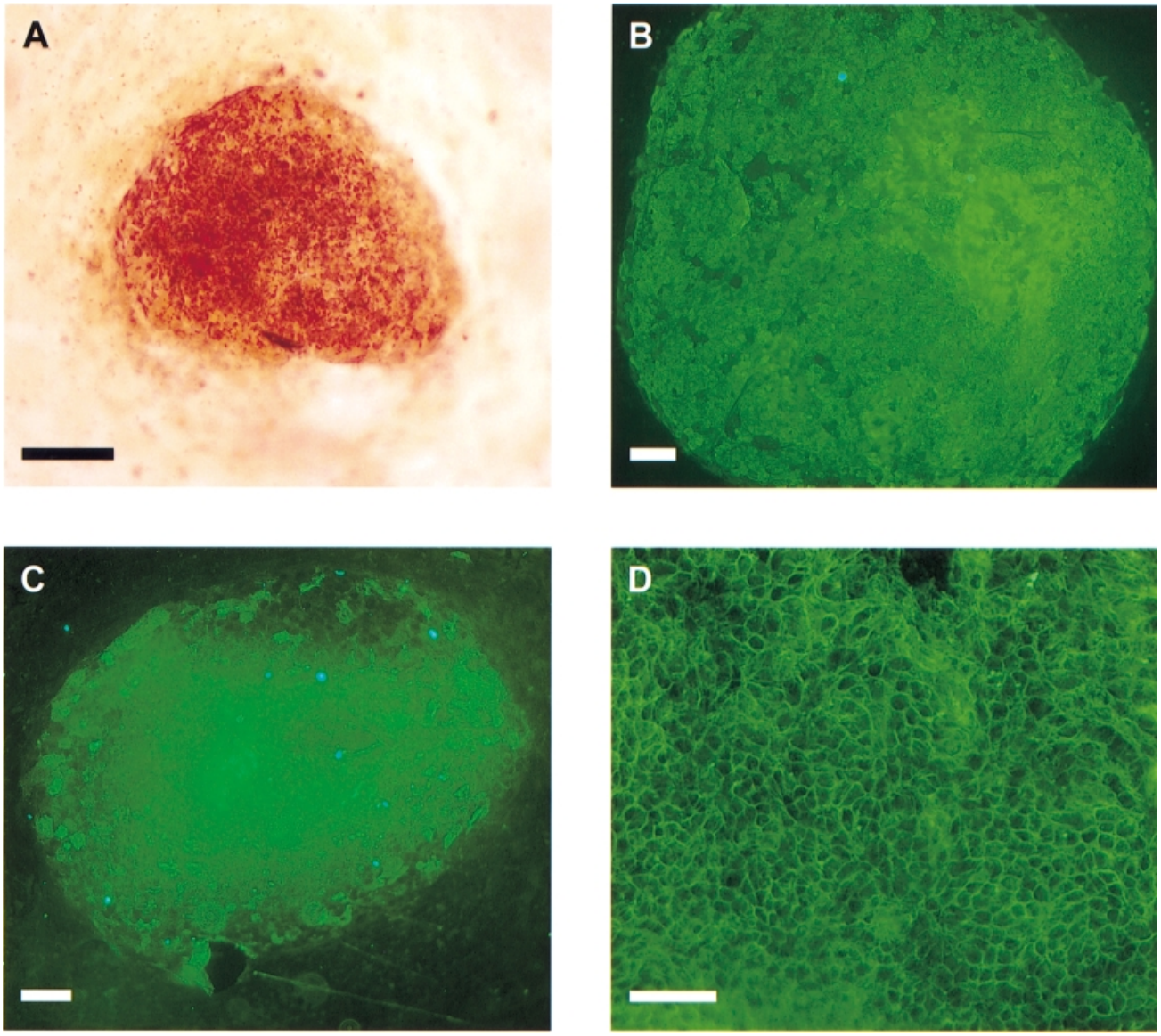

Figure 2. Marker expression in vitrified ES cells. (**A**) ES cell colony showing histochemical staining for alkaline phosphatase. (**B**) ES cell colony stained with antibody TRA1-60. (**C**) ES cell colony stained with antibody GCTM-2. (**D**), ES cell colony stained with antibody MC-813-70 recognizing the SSEA-4 epitope. Scale bars: A–D = 100 μm.

Standard G-banding techniques were used for karyotyping (at passage levels 3 and 16 for HES-1 and HES-2 respectively).

Teratoma formation in severe combined immunodeficient (SCID) mice

At the time of routine passage, clumps of about 200 undifferentiated cells that had originated from vitrified human ES cells were harvested as described above, and injected into the testis of 4- to 8-week-old SCID mice (CB17 strain; Walter and Eliza Hall Institute, Melbourne, Australia; 10–15 clumps/testis). Clumps from each cell line (HES-1 passage level 4 and HES-2 passage level 3 after vitrification) were injected into three mice. At 6–7 weeks later, the resulting tumours were fixed in neutral buffered formalin 10%, embedded in paraffin and examined histologically after haematoxylin and eosin staining.

Results

Human pluripotent ES cells from both cell lines could be successfully recovered and propagated after cryopreservation with conventional slow-rate freezing and rapid thawing methods (Freshney, 1994), and the cells retained their fundamental characteristics. Nevertheless, the efficiency of these standard methods with human ES cells was relatively low. It was possible to recover only 70% (65/93) of the clumps after thawing, and only 16% (15/93) developed after plating into undersized ES cell colonies with a high level of differentiation. Prolonged culture (2 weeks) was required to allow proliferation of undifferentiated cells in the minority of these colonies. In contrast, an improved outcome was observed when the OPS vitrification method was used. All vitrified ES cell clumps that originated from both cell lines (n = 56) were recovered after thawing, and all generated colonies after plating.

In order to evaluate the effect of vitrification on the survival and level of differentiation of ES cells, the area and the extent of differentiation of HES-1 colonies that developed from vitrified clumps was compared with that for non-vitrified

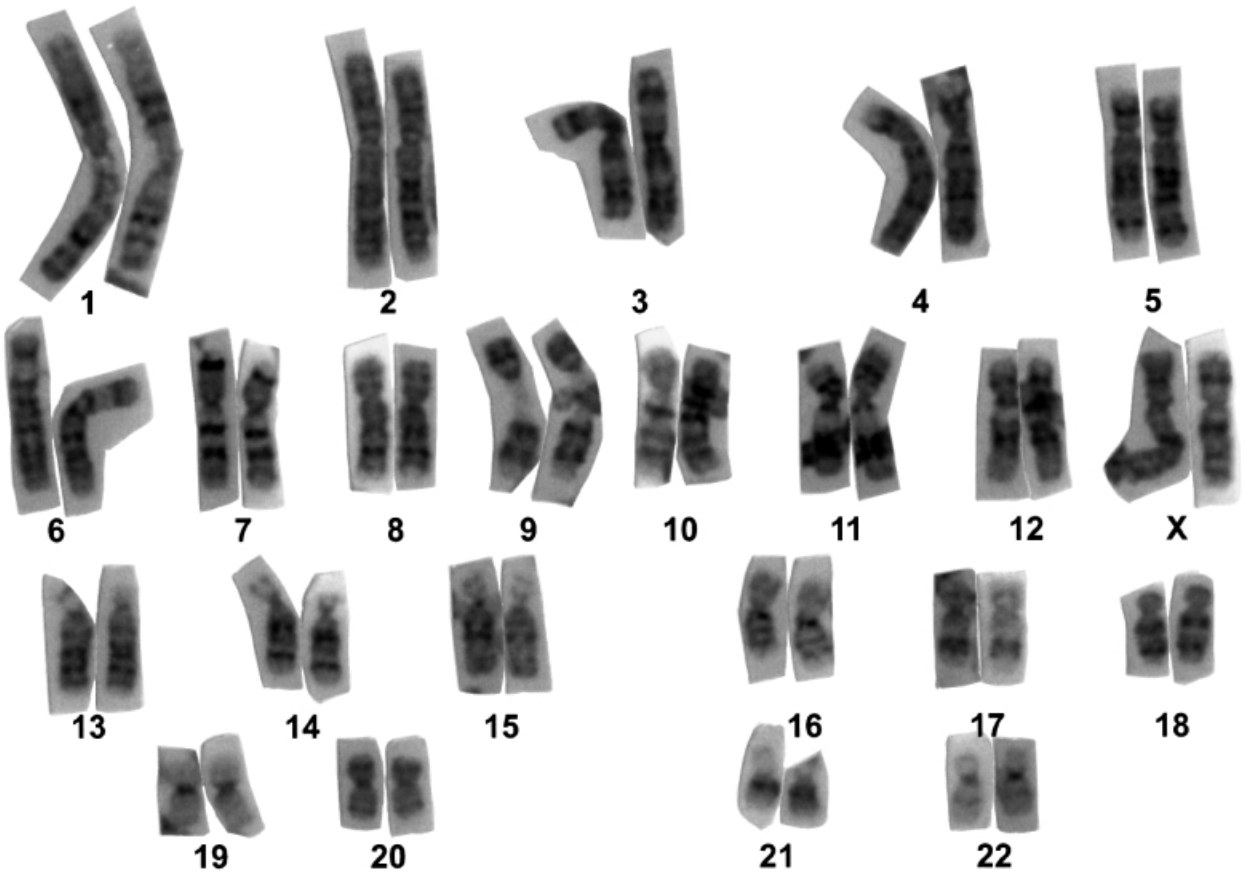

Figure 3. Normal karyotype (46,XX) of vitrified human ES cells (cell line HES-2).

controls. All vitrified clumps (n = 25) were recovered after thawing, and all produced colonies after plating. Vitrification was associated with some cell death, as was evident from the observation of floating dead cells above the colonies at the first day after plating and the significantly reduced mean area of the colonies at days 2 and 7 after plating, compared with control colonies (Table I; Figure 1A and B). However, an additional day in culture was sufficient to overcome the vitrification-induced cell deficit. The mean area of the vitrified colonies at day 8 after plating was similar to the area of the control colonies at day 7 (Table I).

Vitrification was also associated with a significant increase in the level of background differentiation as evaluated at day 7 compared with controls. Nevertheless, the morphological appearance of the colonies improved with additional time in culture. This effect was probably due to the proliferation of undifferentiated cells. At day 9 after plating the trend towards increased level of differentiation in vitrified ES colonies was not-significant when compared with the level of differentiation among day 7 control colonies (Table I).

Vitrified ES cells from both cell lines retained the morphology (Figure 1C and D), the key properties and characteristics of human pluripotent cells. The vitrified cells could be propagated for prolonged periods (19 passages HES-1, 17 passages HES-2). Marker and karyotype analysis were performed on both vitrified cell lines. The vitrified ES cells contained alkaline phosphatase activity (Figure 2A). Immunophenotyping of the vitrified ES cells was carried out using a series of antibodies

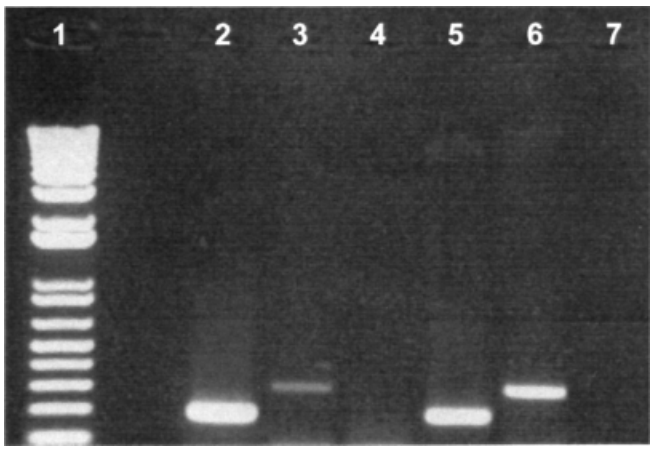

Figure 4. RT-PCR analysis of the expression of Oct-4 and beta-actin in vitrified human ES stem cells. Gel is 1.5% agarose, stained with ethidium bromide. Lane 1, 100 bp DNA ladder; lane 2, human embryonal carcinoma cell culture, beta actin; lane 3, human embryonal carcinoma cell culture, Oct-4 (positive control); lane 4, human embryonal carcinoma cell culture, PCR for Oct-4 carried out with omission of reverse transcriptase; lane 5, vitrified human ES cell culture, beta actin; lane 6, vitrified human ES cell culture, Oct-4; lane 7, vitrified human ES cell culture, PCR for Oct-4 carried out with omission of reverse transcriptase. The beta actin band is 200 bp, and the Oct-4 band is 320 bp.

that detect cell-surface carbohydrates and associated proteins found on human pluripotent cells (Andrews *et al*., 1996; Thomson *et al*., 1998; Reubinoff *et al*., 2000). The ES cells

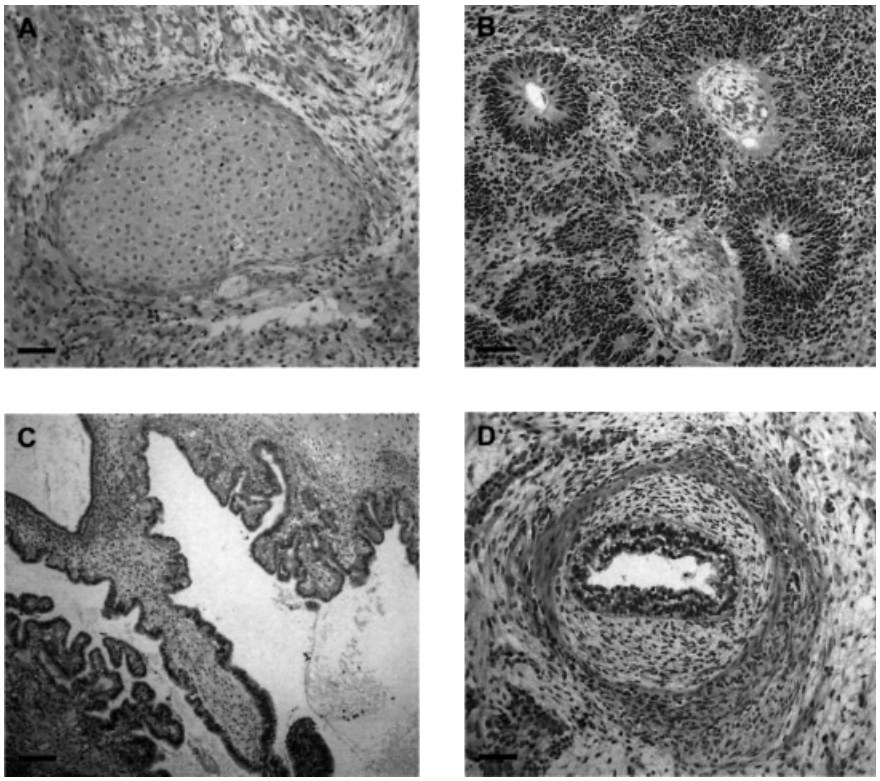

Figure 5. Histology of differentiated elements found in teratomas formed in the testis of severe combined immunodeficient (SCID) mice following inoculation of human ES cell colonies. (A) Cartilage; (B) neural rosettes; (C) choroid plexus; (D) primitive bronchus. Scale bars: A, B and D = 50 μm; C = 100 μm.

reacted positively in indirect immunofluorescence assays with antibodies against the TRA 1-60 carbohydrate epitopes and SSEA-4 (Figure 2B and D). ES cells also reacted with the monoclonal antibody GCTM-2, which detects an epitope on the protein core of a keratan sulphate/chondroitin sulphate pericellular matrix proteoglycan (Pera *et al*., 1988; Badcock *et al*., 1999) found in human embryonal carcinoma cells (Figure 2C). Both cell lines retained their normal female karyotype (Figure 3).

Oct-4 is a POU domain transcription factor the expression of which is limited in the mouse to pluripotent cells, and recent results show directly that zygotic expression of Oct-4 is essential for establishment of the pluripotent stem cell population of the inner cell mass (Nichols *et al*., 1998). Moreover, the level of Oct-4 governs the fate of ES cells, a critical level being required to sustain stem cell self-renewal. Increased expression of Oct-4 induces differentiation into primitive endoderm and mesoderm, while down-regulation of Oct-4 levels results in dedifferentiation to trophectoderm (Niwa *et al*., 2000). It has been shown previously that Oct-4 is also expressed in human ES cells, and that its expression is down-regulated when these cells differentiate (Reubinoff *et al*., 2000). Reverse-transcribed-polymerase chain reaction (RT-PCR) analysis of mRNA isolated from colonies consisting mainly of stem cells, showed that human ES cells retain the expression of Oct-4 after vitrification (Figure 4). The PCR product was cloned and sequenced and shown to be identical to human Oct-4 (not shown).

To confirm that the ES cells retained their pluripotent potential after vitrification, differentiation of the vitrified cells was examined in xenografts. Both HES-1 and HES-2 colonies were inoculated beneath the testis capsule of SCID mice. All mice developed tumours, and in most cases both testes were affected. At autopsy, lesions were observed consisting of cystic masses filled with pale fluid and areas of solid tissue. There was no gross evidence of metastatic spread to other sites within the peritoneal cavity. Histological examination revealed that the lesion had displaced the normal testis, and contained solid areas of teratoma. Embryonal carcinoma was not observed in any lesion. The teratomas from both cell lines contained tissue representative of all three germ layers. Differentiated tissues seen included cartilage, primitive neuroectoderm, choroid plexus, primitive bronchus and glandular structures (Figure 5).

Discussion

This study was undertaken to test the hypothesis that methods which are useful for the cryopreservation of embryos may be also beneficial for the cryopreservation of human ES cells. The two approaches that are mainly used for the cryopreservation of embryos are a modification of the traditional slow-rate freezing method (Whittingham *et al*., 1972; Trounson and Mohr, 1983; Kaufman *et al*., 1995) and vitrification (Rall and Fahy, 1985). Cryopreservation of embryos with the traditional slow-freezing procedure is time consuming and requires an expensive programmable embryo freezer, which is not available in most developmental biology laboratories. Therefore, the choice was

taken to evaluate the efficacy of vitrification, which is a short procedure performed with a simple container of liquid nitrogen.

With the vitrification approach, a glass-like solidification of the freezing solution is achieved by using a high concentration of cryoprotectant and rapid cooling. While this approach can eliminate cell injury due to ice crystal formation, the high concentration of cryoprotectant may induce significant toxic and osmotic damage. The concentrations of cryoprotectants required to achieve vitrification are inversely related to the rate of cooling. Therefore, an increased speed of cooling can lessen the cryoprotectant-induced toxicity, as it minimizes the time of exposure to these toxic compounds and allows their use at reduced concentrations. An increased cooling rate has been recently achieved by plunging small-volume samples held on electron microscopy grids (Martino *et al*., 1996), thin-walled open pulled straws (Vajta *et al*., 1997a) and small nylon loops (Lane *et al*., 1999a) directly into liquid nitrogen. Given the promising results of these approaches with embryos from species that are highly sensitive to cryoinjury (Vajta, 1997b; Lane *et al*., 1999a; Park *et al*., 1999) and with oocytes that are also highly chill-sensitive (Kuleshova *et al*., 1999; Chen *et al*., 2000), the current study sought to evaluate this strategy for the cryopreservation of human ES cells.

The results indicate that vitrification by the OPS method is an effective approach for the cryopreservation of human ES cells. All vitrified clumps of human ES cells were recovered after thawing, and developed into ES cell colonies after plating. The vitrified human ES cells retained the key properties of pluripotent cells, as demonstrated by their normal karyotype, marker expression and the potential to differentiate to derivatives of the three germ layers in xenografts.

It should be noted that vitrification of human ES cells was associated with some cell injury. A significant increase in the levels of cell death and spontaneous differentiation were observed after thawing, though additional proliferation of the ES cells in culture for 2 days was sufficient to overcome these unwanted effects. Therefore, it seems that for the purpose of cryopreservation of cell lines, the practical significance of this cryoinjury is probably negligible. It is possible that alteration of the composition of the vitrification solution in favour of the relatively less toxic cryoprotectant ethylene glycol (Palasz and Mapletoft, 1996), use of a nylon loop instead of the open pulled straw (Lane *et al*., 1999a) and increasing the rate of cooling may reduce the vitrification-induced cell injury.

Increased cooling rate vitrification methods require direct contact between the cell-containing medium and the liquid nitrogen (Martino *et al*., 1996; Vajta *et al*., 1997a; Lane *et al*., 1999a). Although this direct contact carries a potential hazard for transmission of infective agents (Tedder *et al*., 1995), no evidence of any contamination was observed during prolonged cultivation after vitrification. Nonetheless, the potential hazard of contamination may be eliminated by utilizing the OPS vitrification method under sterile conditions (Vajta *et al*., 1998b).

The promising results of cryopreservation of bovine and hamster embryos by using high cooling rate vitrification methods (Vajta *et al*., 1997a; Lane *et al*., 1999a), triggered initial attempts to use these techniques for the cryopreservation of human blastocysts. In a recent report, human blastocysts that were vitrified with a cryoloop did not show any reduction in their ability to re-expand and hatch in culture (Lane *et al*., 1999b). These results indicated that vitrification did not adversely affect the function of human blastocyst trophectodermal cells. The current data further support the possible future use of these methods for the cryopreservation of human blastocysts, as it has been demonstrated that human pluripotent inner cell mass-like cells retained a normal karyotype, and their pluripotent developmental potential after vitrification.

The cryopreservation of human ES cells may serve as an in-vitro model to test and compare the efficacy of various cryopreservation protocols before their implementation in human blastocysts. Nevertheless, while cryopreservation of human ES cells may serve as a reliable model for the pluripotent cells of the inner cell mass, this model will not be totally predictive of the outcome of cryopreservation for the blastocyst, which has a distinct structure and contains trophectoderm in addition to pluripotent inner cell mass cells.

The surface area of the colonies has been used to estimate the number of ES cells, and the morphological appearance of the colonies to evaluate differentiation. While these crude measurements were sufficient for the purposes of the current study, more accurate and objective parameters may be required if ES cells are to be used as an in-vitro screening model. In this regard, flow cytometric assays are currently under development that will allow accurate cell counting and evaluation of the proportion of undifferentiated versus differentiated cells.

In conclusion, the results of the current study indicate that human ES cells may be effectively cryopreserved by using the OPS vitrification method. The key properties of pluripotent cells were maintained after thawing. The efficient cryopreservation approach presented here improves the handling of human ES cell lines, and may be used for the storage of stocks of cells and the establishment of ES cell banks. In addition, it may facilitate the efficient interlaboratory transfer of cells which, in turn, may promote the widespread use of human ES cell lines. The current data also support the possible application of the OPS or similar vitrification methods for the cryopreservation of human blastocysts.

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