Novel cryopreservation method for dissociated human embryonic stem cells in the presence of a ROCK inhibitor

R. Martin-Ibañez1,2,5, C. Unger3, A. Strömberg2, D. Baker4, JM. Canals1 and O. Hovatta2

1Departament de Biologia Cel·lular, Immunologia i Neurociències, Facultat de Medicina, IDIBAPS, Universitat de Barcelona and Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED) E-08036, Barcelona, Spain; 2Department of Clinical Science, Intervention and Technology, Karolinska Institutet, Karolinska University Hospital Huddinge SE 141 86, Stockholm, Sweden; 3Department of Medicine, Karolinska Institutet, Karolinska University Hospital Huddinge SE 141 86, Stockholm, Sweden; 4Sheffield Regional Cytogenetics Service, Sheffield Children’s Trust, Western Bank S10 2TH, Sheffield, UK

5Correspondence address. E-mail: r.martin@ub.edu

BACKGROUND: Human embryonic stem cells (hESCs) have potential use in clinical therapy and regenerative medicine. One of the major challenges regarding the application of these cells is the development of an efficient cryopreservation protocol, since current methods, which include slow-freezing–rapid thawing and vitrification of colonies in suspension, present poor viability and high differentiation rates. Dissociated hESC suspensions do not survive cryopreservation because they are susceptible to apoptosis upon cell detachment and dissociation. A selective Rho-associated kinase (ROCK) inhibitor has been reported to increase the survival of dissociated hESCs and their cloning efficiency.

METHODS AND RESULTS: Here, we describe a novel method for dissociated hESCs cryopreservation in the presence of the ROCK inhibitor Y-27632. The addition of this inhibitor to the freezing and post-thawing medium significantly increased the survival rate and efficiency of colony formation. Moreover, the hESC colonies obtained after the cryopreservation in the presence of the ROCK inhibitor showed a very low rate of differentiation and a reduced time of recovery. After prolonged culture of frozen–thawed dissociated hESCs, the characteristic properties of pluripotent cells were observed, including normal karyotype, morphological features, marker expression (SSEA-4, TRA-1-60, TRA-1-81 and Oct-4) and the potential to differentiate into derivatives of all three germ layers after embryoid bodies formation.

CONCLUSION: This novel method for the cryopreservation of dissociated hESCs may reduce the time required to amplify frozen stocks, and facilitate not only the storage of large numbers of hESCs but also the widespread use of these cells in regenerative medicine.

Keywords: human embryonic stem cells; Y-27632; cryopreservation; dissociation; survival

Introduction

hESCs have generated much interest over recent years because they hold great potential as a source of stem cells for cell therapy and regenerative medicine (Thomson et al., 1998; Trounson and Pera, 2001; Klimanskaia et al., 2008). However, this will not be fully realized until the techniques for the handling, manipulation and cryopreservation of these cells have been improved. In this context, the establishment of an efficient hESC cryopreservation protocol with limited loss in viability and low differentiation rates would be extremely valuable for the development and widespread use of these cell lines.

In general, two techniques have been applied for hESC cryopreservation. The conventional slow-freezing and rapid-thawing method using dimethylsulfoxide (DMSO) as a cryoprotectant is the most common (Grout et al., 1990; Meryman, 2007). Although this procedure is efficient for the cryopreservation of mouse embryonic stem cells (mESCs), it has shown poor performance for undifferentiated hESCs, most of which either differentiate or die (Reubinoff et al., 2001; Richards et al., 2004).

In contrast, vitrification of hESCs by the open pulled straw method using high cryoprotectant concentrations together with flash-freezing in liquid nitrogen leads to much higher cell survival rates (Reubinoff et al., 2001; Richards et al., 2004; Li et al., 2008). However, vitrified hESC colonies still suffer from high levels of cell death, slow growth rates and high levels of differentiation. Moreover, these protocols are tedious to perform manually and are clearly unsuited for handling the bulk amounts of hESCs required for clinical and non-clinical applications (Heng et al., 2006).

Given their susceptibility to apoptosis upon cellular detachment and dissociation, hESCs are generally split and cryopreserved in small aggregates (100–200 cells). hESCs undergo
massive cell death after complete dissociation, and cloning efficiency is generally low (Thomson et al., 1998; Amit et al., 2000). However, the application of a selective Rho-associated kinase (ROCK) inhibitor (Ishizaki et al., 2000; Hu and Lee, 2005), Y-27632, to hESCs markedly diminishes dissociation-induced apoptosis, thereby increasing cloning efficiency (Watanabe et al., 2007). In view of these results, we examined the effects of the ROCK inhibitor Y-27632 on the cryopreservation of dissociated hESCs by the slow-rate freezing and rapid-thawing method. We describe an efficient technique that allows the cryopreservation of large numbers of hESCs and ensures high cell survival rates and hardly any differentiation, which in turn gives rise to low recovery times.

Materials and Methods

hESC maintenance culture

Two hESC lines were used in this study: HS207 and HS401. Both lines were derived from the inner cell mass of 5/6-day-old pre-implantation blastocyst-stage embryos obtained after in vitro fertilization at the Fertility Unit of the Karolinska University Hospital Huddinge, Karolinska Institutet, Sweden. The Ethics Committee of the Karolinska Institutet gave approval for the derivation, expansion and differentiation of the hESC lines. hESC colonies were cultured and maintained as previously described (Hovatta et al., 2003; Inzunza et al., 2005). Briefly, colonies were cultured on a feeder layer of human foreskin fibroblasts (CRL-229; ATCC, Manassas, VA, USA) mitotically inactivated by irradiation (40 Gy), in Knockout Dulbecco’s Modified Eagle’s medium supplemented with 20% Knockout serum replacement, 2 mM Glutamax, 0.5% penicillin–streptomycin, 1% non-essential amino acids (all from Gibco Invitrogen Corporation, Oxon, UK), 0.5 mM 2-mercaptoethanol (Sigma–Aldrich Co., St. Louis, USA) and 8 ng/ml bFGF (R&D Systems, Oxon, UK) at 37°C in 5% CO₂.

hESC lines were propagated as described previously (Inzunza et al., 2005). hESC colonies were split mechanically at 6- to 8-day intervals. Colonies, containing on average 20 000 cells, were cut into 6–8 portions and then transferred onto fresh feeder cells.

Splitting of dissociated hESCs in the presence a ROCK inhibitor

hESCs lines were split, dissociating into single cells in the presence of a ROCK inhibitor as previously described (Watanabe et al., 2007), with some modifications. In brief, 10 μM of the ROCK inhibitor Y-27632 (Merck Chemicals, Ltd., Nottingham, UK) were added to the culture medium 1 h before detaching the cells from the feeder layer. Cells were detached from this layer by the treatment with TrypLE™ Express (Gibco Invitrogen Corporation) at 37°C for 5 min, followed by flushing with a pipette several times in order to detach single hESCs from the feeders. Dissociated hESCs were seeded at low (1500 cells/cm²) and high density (15 000 cells/cm²) onto fresh feeder layers in the presence of the ROCK inhibitor Y-27632 during the first day of culture.

The efficiency of colony formation was determined as the percentage of hESC colonies formed per number of hESCs seeded.

Cryopreservation of dissociated hESCs

Confluent plates obtained by splitting hESCs into single cells in the presence of Y-27632 were harvested as described above. Dissociated hESCs were then transferred to the freezing medium: 90% hESC culture medium without bFGF supplemented with 10% DMSO (Sigma–Aldrich Co.). Between 30 000–100 000 hESCs were loaded into a cryovial (Nunc A/S, Roskilde, Denmark) and frozen (at the rate of −1°C/min until −80°C) using a freezing container (Nalgene Europe Ltd., Hereford, UK), and then stored in liquid nitrogen. One to two weeks after cryopreservation, hESCs were rapidly thawed in a water bath at 37°C, washed twice in hESC culture medium and plated on a fresh feeder layer. hESC colonies recovered after thawing were split mechanically, dividing into portions, as described for the maintenance culture.

To study the effect of ROCK inhibition during cryopreservation of dissociated hESCs, 10 μM of Y-27632 was added to the freezing and/or post-thawing medium establishing four different conditions of study (Table I).

As a control condition, we used hESCs that had been split into single cells twice in the presence of Y-27632 and then returned to mechanical splitting as for the frozen–thawed hESCs.

Assessment of hESC viability and adhesion properties after cryopreservation

We counted the number of undifferentiated colonies formed 7–10 days after seeding 30 000 cryopreserved hESCs in each of the conditions tested (1−/−, 2+/−, 3−/+ and 4+/+) and in the control condition.

The level of differentiation for each colony was determined using inverted/phase-contrast microscopy on the basis of morphological appearance: colonies formed by small cells with a high nucleus:cytoplasm ratio were regarded as undifferentiated, whereas colonies with large cells with abundant cytoplasm were considered differentiated. In addition, morphological appearance was assessed by Oct4 immunocytochemistry.

<p>| Table I. Conditions established for the study of the effect of Y-27632 in the cryopreservation of dissociated hESCs in the basis of the presence (+) or absence (−) of this ROCK inhibitor in the freezing and post-thawing medium. |</p>
<table>
<thead>
<tr>
<th>Condition referred to as</th>
<th>Presence of Y-27632 in the freezing medium</th>
<th>Presence of Y-27632 in the post-thawing medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Y-27632 was added to the post-thawing medium only the first day after thawing.
Immunocytochemical characterization

Immunocytochemical characterization was carried out for colonies obtained from dissociated frozen–thawed hESCs and for colonies obtained by mechanical splitting at passages 2, 5 and 10 after cryopreservation. Immunostaining was performed as previously described (Strom et al., 2007), with some modifications. In brief, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature, washed in PBS and blocked for 30 min with 30% fetal bovine serum. Permeabilization (not for surface markers) was done using 30% blocking buffer consisting of 0.1% saponin (S4521; Sigma–Aldrich Co.) in PBS for 1 h at room temperature. Primary antibodies: goat anti-Oct4 polyclonal IgG (1:200), mouse anti-TRA-1-60 monoclonal IgG (1:200), mouse anti-TRA-1-81 monoclonal IgG (1:200), mouse anti-Nestin human-specific IgG (1:200) (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rabbit anti-Nanog polyclonal IgG (1:100), mouse anti-sSEA-3 (1:200) and mouse anti-sSEA-4 monoclonal IgG (1:200) all from Chemicon, were added in PBS overnight at 4°C, and the cells were washed three times with PBS to remove any unbound antibodies. The secondary antibodies, FITC-conjugated bovine anti-goat IgG, Cy3-conjugated goat anti-mouse IgG, FITC-conjugated bovine anti-goat IgG and Rhodamine conjugated goat anti-rabbit IgG (all from Chemicon) were diluted 1:200 in PBS and applied to the cells for 1–2 h at room temperature in the dark. After three washes in PBS, cell nuclei were counterstained with Hoechst B2261 (Sigma–Aldrich Co.) for 10 min.

We used human foreskin fibroblasts as negative control cells. Negative controls not treated with primary antibodies resulted in no staining.

Stained cells were analyzed with an Olympus IX71 inverted microscope (Olympus Sverige AB, Solna, Sweden) and images were acquired with an Olympus DP71 camera and the Soft Imagine Cell F version 2.6 Software (Olympus Sverige AB).

RT-PCR

To study the non-differentiated state of the hESC colonies obtained after thawing (referred to as passage 0) and at passages 2, 5 and 10, we analyzed the mRNA levels of Oct4 expression by RT-PCR, as previously described (Martin-Ibanez et al., 2007), with some modifications. In brief, total RNA from hESCs at distinct passages after freezing–thawing was extracted using High Pure RNA Tissue Kit (Roche Diagnostics Scandinavia, AB, Sweden). Total RNA (500 ng) was used to synthesize cDNA using random primers and the StrataScript® First Strand cDNA Synthesis System (STRATEGENE®). The cDNA synthesis was performed at 42°C for 60 min in a final volume of 20 μl, following the manufacturer’s instructions. The cDNA was then analyzed by PCR using the following TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA): oct4, POU5F1 Hs00742896_s1 and 18S, Hs99999901_s1.

The RT-PCR data were analyzed using the MxPro™ QPCR analysis software version 3.0 (STRATEGENE®). Final quantification was performed using the Comparative Quantitation analysis and is reported as n-fold difference compared with undifferentiated control hESCs cDNA following normalization to 18S control gene. Q-PCR assays were performed in triplicate and repeated in at least three independent experiments for lines HS207 and HS401. To provide negative controls and exclude contamination by genomic DNA, the reverse transcriptase was omitted in the cDNA synthesis step, and the samples were subjected to the PCR in the same manner with each primer.

Testing pluripotency in vitro

Embryoid bodies (EBs) were formed as previously described (Strom et al., 2007) by culturing aggregates of hESCs in suspension in hESC culture medium without bFGF for 3 weeks before harvesting. The presence of tissues originating from the three embryonic germ layers was demonstrated using immunocytochemistry and RT-PCR.

For immunocytochemistry, EBs were attached to Matrigel-coated plates for 1 day, fixed for 1 h with paraformaldehyde 4% and immunostained as described for hESCs with markers of the three embryonic germ layers: Bone morphogenetic protein-4 (BMP-4) for mesoderm (Novoceastra Laboratories Ltd, Newcastle upon Tyne, UK), Nestin for ectoderm (Chemicon) and Alpha-fetoprotein (AFP) for endoderm (Sigma–Aldrich Co.). EBs were counterstained with Hoechst (Sigma–Aldrich Co.) for nuclear staining.

To test pluripotency, we used RT-PCR to determine the expression of the same markers: nestin, BMP-4 and AFP for EBs obtained from cryopreserved lines HS207 and HS401. Using the same protocol described above for RNA extraction and cDNA synthesis, the analysis was done by PCR using the following TaqMan® Gene Expression Assays: BMP-4 (Hs00370078_m1), AFP (Hs00173490_m1) and Nestin (Hs00707120_s1). Reactions were as follows: 35 cycles of a two-step PCR (95°C for 30 s and 60°C for 1 min) after initial denaturation (95°C for 10 min). An additional elongation step of 10 min at 60°C was also done. PCR products were visualized in a 2% agarose gel. To provide negative controls and exclude contamination by genomic DNA, the reverse transcriptase was omitted in the cDNA synthesis step, and the samples were subjected to the PCR in the same manner with each primer. No bands were obtained for the negative controls.

Karyotyping

Karyotyping of cell lines HS207 and HS401 was carried out using standard G-banding techniques at passage 10. Samples of cells were treated with colcemid KaryoMAX (0.1 μg/ml; Gibco Invitrogen Corporation) for up to 4 h, followed by dissociation with TrypLE Express (Gibco Invitrogen Corporation). The cells were pelleted via centrifugation and re-suspended in pre-warmed 0.0375 M KCl hypotonic solution and incubated for 10 min. Following centrifugation, the cells were re-suspended in fixative (3:1 methanol:acetic acid). Metaphase spreads were prepared on glass microscope slides and G-banded by brief exposure to trypsin and stained with 4:1 Gurr’s/Leishmann’s stain (Sigma–Aldrich Co.). A minimum of 10 metaphase spreads were analysed and a further 20 were counted.

Statistical analysis

All experiments were conducted in triplicate in three–four independent cultures considered as independent experiments. Results were expressed as the mean ± standard error of the mean (SEM). The statistical analysis was done using the Student t-test or one way ANOVA depending on the number of groups compared. In this way, we used Student’s t-test to compare two unpaired groups when we were studying: (i) the efficiency of colony formation in the presence or absence of ROCK inhibitor, (ii) the percentage of cell survival and (iii) the percentage of hESCs not attached, after cryopreservation in the presence or absence of the ROCK inhibitor Y-27632. On the other hand, we used one way ANOVA followed by a Bonferroni’s post hoc test to compare three or more unmatched groups. This is the case of the comparison between the number of colonies formed after hESCs cryopreservation in the four conditions studied (Conditions 1−/−, 2+/−, 3−/+ and 4+/+).

Results

ROCK inhibitor allowed single-cell splitting of dissociated hESC lines HS207 and HS401

To develop an efficient cryopreservation protocol for hESCs, we studied the effects of the ROCK inhibitor Y-27632 on the
cryopreservation of dissociated hESCs. With this aim and taking into account that we failed to split our cell lines by single cell dissociation in standard conditions, we first determined the efficiency of colony formation of dissociated hESCs (based on the ratio of hESC colonies formed per initially seeded hESCs) in the presence of Y-27632 for lines HS207 and HS401 (Fig. 1). We pre-treated hESCs for 1 h with 10 μM Y-27632 and after complete dissociation, we seeded the cells on a human feeder layer at low density (1500 cells/cm²). After 6–8 days, many large colonies formed from the Y-27632-treated dissociated hESCs while few or no colonies were observed in untreated dissociated hESCs (Fig. 1A). Thus, reproducing Watanabe’s results (Watanabe et al., 2007), we observed that Y-27632 increased the efficiency of colony formation of dissociated hESCs for both cell lines compared to the untreated control condition (Fig. 1A; HS207: 5.87 ± 1.01 and 0.27 ± 0.09%; HS401: 7.17 ± 0.22 and 0.02 ± 0.01%; in the presence and absence of Y-27632, respectively). Moreover, the colonies obtained from dissociated hESCs in the presence of the ROCK inhibitor presented undifferentiated morphological features, such as well-defined borders and small cells with high nuclear:cytoplasm ratios (Fig. 1B). They expressed standard undifferentiation-markers (Oct4, SSEA-4, TRA-1-60 and TRA-1-81; data not shown).

Cryopreservation of dissociated hESCs in the presence of the ROCK inhibitor Y-27632
We next examined the effects of the ROCK inhibitor Y-27632 on the cryopreservation of dissociated hESCs by the slow-freezing rapid-thawing method (see scheme of the procedure in Fig. 2).

First, we determined the protecting effect of Y-27632 when present in the freezing medium (Fig. 3A). We measured the survival of hESCs cryopreserved in the presence and absence of this ROCK inhibitor by counting the number of live cells before and after freezing using a haemocytometer. Cell viability was assessed by the Trypan Blue exclusion method (Fig. 3A). Treatment with Y-27632 protected dissociated hESCs during cryopreservation for HS207 and HS401 (Fig. 3A; cell survival: HS207: 50.4 ± 1.7 and 33.6 ± 2.2%; HS401: 56.4 ± 7.2 and 30.5 ± 5.2%; in the presence and absence of Y-27632, respectively).

Given the role of Y-27632 in cell adhesion, we next analyzed the number of frozen–thawed hESCs not attached to the feeder layer 48 h after seeding in the presence or absence of this ROCK inhibitor in the post-thawing medium. We counted the number of cells in suspension in the medium using a haemocytometer chamber and observed that Y-27632 increased cell adhesion as there was a reduction in the number of unattached cells for both cell lines (Fig. 3B). The viability of unattached hESCs determined by the Trypan Blue exclusion method was affected (data not shown).

Having demonstrated that Y-27632 increased not only the survival but also the adhesion of frozen–thawed dissociated hESCs, we then studied the effect of this inhibitor on colony formation after thawing. With this purpose, we established four experimental conditions on the basis of the presence (+) or absence (−) of Y-27632 in the freezing and post-thawing medium (Conditions 1−/−, 2+/−, 3−/+ and 4+/+; see Materials and Methods). Frozen–thawed hESCs under these four conditions were seeded and allowed to grow and 7–10 days after the number of colonies was determined (Fig. 3C and D). The cryopreservation of dissociated hESCs was not achieved in the absence of the ROCK inhibitor since almost no colonies were obtained in Condition 1−/−. However, Y-27632 treatment during the freezing process (Condition 2+/−) increased the number of colonies formed compared with Condition 1, although not significantly. Moreover, the
Immediately after the cryopreservation of dissociated hESCs, we used immunocytochemistry to characterize the colonies obtained. Our results showed that all the colonies expressed the surface markers SSEA-4, SSEA-3, TRA-1-60 and TRA-1-81 and the nuclear marker Nanog (Fig. 4).

To assess the maintenance of the undifferentiated state of frozen–thawed dissociated hESCs along time, we next analyzed the levels of Oct4 expression using RT-PCR at a range of time points after cryopreservation (after thawing (Passage 0), Passages 2, 5 and 10). The frozen–thawed dissociated hESCs maintained their undifferentiated state over time since no differences were found between the levels of Oct4 expression at any time point analyzed for either HS207 or HS401 when compared with undifferentiated control hESC colonies, which were maintained in standard conditions (Fig. 5A). Consistent with this result, all the colonies obtained 10 passages after cryopreservation expressed the nuclear and surface markers Oct4, Nanog, SSEA-4, TRA-1-60 and TRA-1-81 (Fig. 5B).

Karyotype analysis was performed in hESCs 10 passages after cryopreservation to demonstrate the absence of chromosomal abnormalities. The results showed for both cell lines, a normal male 46, XY karyotype (Fig. 6A).

Finally, we studied the pluripotency of hESCs in vitro after cryopreservation by examining EB formation from HS207 and HS401 at Passages 10–15. Tissue components expressing markers of the three germ layers, such as AFP for endoderm, Nestin for ectoderm and BMP-4 for mesoderm, were identified by RT-PCR (Fig. 6B) and immunocytochemistry (Fig. 6C).

Discussion

hESCs are colony-forming cells that spontaneously differentiate and present poor survival rates when dispersed into single cells (Thomson et al., 1998; Amit et al., 2000). These features explain why traditional cryopreservation of single trypsinized cells using the conventional slow-freezing–rapid-thawing method in medium containing 10% DMSO is unsuitable for these stem cells (Reubinoff et al., 2001; Richards et al., 2004). However, our results demonstrate that cryopreservation of dissociated hESCs is feasible in the presence of the ROCK inhibitor Y-27632. Furthermore, the novel cryopreservation method we describe allows the freezing of large numbers of hESCs with a high efficiency of colony formation after thawing, and very low differentiation rates, thereby giving rise to a low time of recovery compared with established methods (Reubinoff et al., 2001; Ji et al., 2004; Richards et al., 2004; Zhou et al., 2004; Heng et al., 2005).

To establish this efficient protocol for the cryopreservation of dissociated hESCs, we first demonstrated the capacity of the hESC lines HS207 and HS401 to form colonies when dissociated into single cells in the presence of the ROCK inhibitor Y-27632, an observation consistent with results described by Watanabe et al. (2007). The efficiencies of colony formation reported in our study (around 5–7%) are lower than those described previously (around 18–27%; Watanabe et al., 2007). However, these differences can be attributed to the...
Figure 3: Cryopreservation of dissociated hESC is feasible in the presence of Y-27632.

(A) Survival of frozen–thawed hESCs in the presence or absence of Y-27632, expressed as the percentage of live cells after thawing with respect to the total number of frozen hESCs, for cell lines HS207 and HS401. (B) Percentage of unattached hESCs 48 h after thawing in the presence or absence of Y-27632 for cell lines HS207 and HS401. (C, D) Number of colonies obtained after thawing of dissociated cryopreserved hESCs in the four conditions established on the basis of the presence or absence of Y-27632 in the freezing and post-thawing medium (Conditions 1−/−, 2+/−, 3−/+ and 4+/+) for cell lines HS207 and HS401. Results are expressed as the mean of 3–4 independent experiments and error bars represent the standard error of the mean (SEM). Statistical analysis was performed using A, B: Student’s t-test. ***p < 0.001 *p < 0.05 compared with the control condition not treated with Y-27632. (C, D) One-way ANOVA followed by Bonferroni’s post hoc test. $p < 0.05 compared with Condition 3−/+; $p < 0.01, $$p < 0.005 compared with Condition 2+/−; **p < 0.01, ***p < 0.005 compared with Condition 1−/−. (E) Representative fluorescent photomicrographs at low and high magnification of Oct4-positive hESC colonies obtained after cryopreservation in the presence of the ROCK inhibitor. Hoechst counterstaining is shown for both magnifications. Scale bar: 100 and 50 μm for low and high magnification, respectively.
Figure 4: Cryopreserved dissociated hESCs in the presence of Y-27632 expressed undifferentiated markers. Immunocytochemical characterization of hESC colonies obtained after single cell cryopreservation in the presence of the ROCK inhibitor Y-27632 at Passage 0. All colonies were positive for the surface markers SSEA-4 (A), TRA-1-60 (B), TRA-1-81 (C), SSEA-3 (D) and the nuclear marker Nanog (E). Hoechst counterstaining is shown for all markers. Scale bar: 100 μm.
Figure 5: hESCs maintained their undifferentiated state over time after single cell cryopreservation in the presence of Y-27632. (A) RT-PCR analysis of the levels of Oct4 expression in hESCs colonies obtained at Passages 0, 2, 5 and 10 after cryopreservation for cell lines HS207 and HS401. Results are normalized to the levels of expression obtained from undifferentiated control hESCs colonies considered 100%. Results are expressed as the mean of 3–4 independent experiments and error bars represent standard error of the mean (SEM). (B) Representative photomicrographs showing hESCs colonies obtained 10 passages after cryopreservation positive for the nuclear markers: Nanog and Oct4 and the surface markers: SSEA-4, TRA-1-60 and TRA-1-81. Hoechst counterstaining is shown for all markers. Scale bar: 100 μm.
Figure 6: Cryopreserved dissociated hESCs retained key properties of pluripotent cells. (A) Karyotype analysis of hESC lines HS207 and HS401 performed at passage 12–10, respectively, using G-band method. Representative pictures show normal 46 XY karyotype for both cell lines. (B and C) In vitro differentiation of hESCs by EB formation at passage 10 after cryopreservation. (B) PCR analysis of the expression of markers of the three embryonic germ layers: Nestin, AFP and BMP-4 as ectodermal, endodermal and mesodermal markers, respectively, in differentiated EBs for both cell lines HS207 and HS401. Negative controls did not showed any band. (C) Representative photomicrographs showing differentiated EBs expressing mesodermal (BMP-4), endodermal (AFP) and ectodermal (Nestin) markers. Hoechst counterstaining is shown for all markers. Scale bars: 50 μm.
distinct hESC lines used in these studies. Our cell lines, HS207 and HS401, could not be split into single cells in the absence of the ROCK inhibitor Y-27632 whereas Khes-1, the hESC line used by Watanabe et al., showed a low but evident efficiency of colony formation. Secondly, the differences can also be attributed to variations between culture and cell-passaging protocols. For instance, in order to have a xeno-free culture for future clinical applications (Hovatta et al., 2003; Mallon et al., 2006; Rajala et al., 2007), we grew hESCs on human feeder cells instead of mouse feeder cells.

The loss of viability of cryopreserved hESCs during freeze–thawing using conventional slow-cooling protocols has been attributed mainly to apoptosis (Heng et al., 2006). Given that ROCK inhibitors show anti-apoptotic activity (Shi and Wei, 2007; Watanabe et al., 2007; Koyanagi et al., 2008; Lingor et al., 2008), we studied the protecting effect of Y-27632 during the cryopreservation of dissociated hESCs. Our results showed that the addition of Y-27632 to only the freezing medium, although increasing hESC survival, was not enough to allow the formation of a significant number of colonies compared with the control condition, which was not subjected to Y-27632 treatment. These results are consistent with the findings of a previous study in which the addition of a caspase inhibitor Z-VAD-FMK to the freezing solution alone did not give rise to any significant enhancement of post-thaw colony progression (Heng et al., 2007). In contrast, the addition of Y-27632 to only the post-thawing medium increased the formation of hESC colonies significantly, suggesting that the protective effect of this ROCK inhibitor is more critical in this step of cryopreservation. Interestingly, the highest efficiency of colony formation was achieved by the addition of the Y-27632 during both freezing and post-thawing, thereby having an additive effect.

Although the presence of Y-27632 during the cryopreservation of dissociated hESCs allowed the formation of a large number of colonies, the efficiency of this formation was reduced about 5-fold compared with that for dissociated hESCs split in control conditions without cryopreservation. This difference can be explained, in part, by an overestimation of the number of live hESCs counted after thawing by the Trypan Blue exclusion method as this method does not allow the detection of apoptotic hESCs in the culture. Another explanation could be that the stress suffered by hESCs submitted to cryopreservation reduces their colony-forming capacity. However, although the efficiency of colony formation was reduced after hESCs cryopreservation, the protocol described here still provides a higher percentage of recovery over established methods (Reubinoff et al., 2001; Richards et al., 2004) if we consider that between 100 and 200 colonies can be obtained from 20 000 cryopreserved hESCs (approximate number present in one hESC colony).

The ROCK inhibitor Y-27632 has also been implicated in mechanisms of cell adhesion for a number of cell types (Anderson et al., 2002; Honjo et al., 2007). In agreement with these studies, we found an increase in the number of hESCs attached to the feeders after thawing in the presence of Y-27632 compared with the control condition. This result suggests that the enhancement of colony formation observed after hESC cryopreservation in the presence of this ROCK inhibitor is due not only to an anti-apoptotic effect but also to an increase in cell adhesion. Further experiments are required to clarify whether the adhesion effect is a consequence of the pro cell survival effect or whether the ROCK inhibitor exerts a dual role.

Other main challenges in the design of a hESC cryopreservation method are the reduction of the differentiation rates and time of recovery, two of the problems encountered when using traditional cryopreservation protocols that include slow-freezing–rapid thawing and vitrification (Reubinoff et al., 2001; Ji et al., 2004; Richards et al., 2004; Heng et al., 2007). In the present study, we describe a novel cryopreservation protocol which almost completely removes differentiation since we failed to detect any differentiated colony with undefined borders and low nuclear:cytoplasm ratios in our morphological analysis (Reubinoff et al., 2001; Richards et al., 2004). Moreover, all colonies expressed nuclear and surface markers such as Oct4, Nanog, SSEA-4, TRA-1-81 confirming the undifferentiated state observed morphologically (Hoffman and Carpenter, 2005). Furthermore, our method also led to a significant decrease in the time of recovery after cryopreservation because small numbers of dissociated cryopreserved hESCs (3 × 10^4–5 × 10^5 cells) produce confluent plates of undifferentiated colonies that are ready to be used for distinct purposes 7–10 days after thawing. In addition, this cryopreservation method does not produce chromosomal abnormalities in hESC neither affects their potential to differentiate towards all three germinal lines.

In conclusion, we have established an efficient method for the cryopreservation of large numbers of dissociated hESCs. This technique may facilitate hESC storage in stem cell banks and the widespread use of these cells for research activities and clinical applications.

Author Roles
R.M-I., conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, critical review and final approval of manuscript.
C.U., collection and/or assembly of data, data analysis and interpretation, critical review and final approval of manuscript.
A.M.S., collection and/or assembly of data, interpretation of results, critical review and final approval of manuscript.
D.B., collection and/or assembly of data, data analysis and interpretation, critical review and final approval of manuscript.
J.M.C., data analysis and interpretation, critical review and final approval of manuscript.
O.H., conception and design, data analysis and interpretation, critical review and final approval of manuscript.

Acknowledgements
We thank M.T. Muñoz and C. Herranz for technical support. We also thank Robin Rycroft for English language revision.

Funding
This study was supported by grants from the Ministerio de Educación y Ciencia (SAF2006-04202, J.M.C.; Spain), the
Ministerio de Sanidad y Consumo [RETICS (Red de Terapia Celular), J.M.C.; Spain] and the Swedish Research Council (O.H.; Sweden). R.M.-I. is a fellow from ESTOOLS under the European Union’s Sixth Framework Programme.

References


Submitted on May 5, 2008; resubmitted on July 17, 2008; accepted on July 24, 2008.