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ORIGINAL ARTICLE *Embryology*

A simple and efficient cryopreservation method for feeder-free dissociated human induced pluripotent stem cells and human embryonic stem cells

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BACKGROUND: An essential prerequisite for the future widespread application of human induced pluripotent (hiPSCs) and embryonic stem cells (hESCs) is the development of efficient cryopreservation methods to facilitate their storage and transportation.

METHODS: We developed a simple and effective freezing/thawing method of single dissociated hESCs and hiPSCs in a feeder-free culture in the presence of Rho-associated kinase (ROCK) inhibitor Y-27632.

RESULTS: Exposure to ROCK inhibitor Y-27632 in freezing solution alone does not significantly enhance the post-thaw survival rate of single dissociated hESCs and hiPSCs. However, when ROCK inhibitor was added to both pre- and post-thaw culture media, there was an enhancement in the survival rate, which further increased when ROCK inhibitor was added to Matrigel as well. Under these treatments, hESCs and hiPSCs retained typical morphology, stable karyotype, expression of pluripotency markers and the potential to differentiate into derivatives of all three germ layers after long-term culture.

CONCLUSIONS: This method is an effective cryopreservation procedure for single dissociated hESCs in feeder-free culture, which is also applicable for single dissociated hiPSCs using a ROCK inhibitor. The cloning efficiency of hiPSCs and hESCs improves when ROCK inhibitor is added both in Matrigel and in medium in comparison with conventional addition to medium. Therefore, we believe this method would be useful for current and future applications of the pluripotent stem cells.

Key words: human embryonic stem cells / human induced pluripotent stem cells / ROCK inhibitor Y-27632 / culture / cryopreservation

Introduction

The derivation of human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs) (Thomson *et al*., 1998) catalyzed a huge increase in interest in the potential therapeutic applications of these cells as tools to study human development and genetic diseases, in toxicological and pharmaceutical applications, and for *in vitro* disease modeling (reviewed in Nishikawa *et al*., 2008). iPSCs are the product of somatic cell reprogramming into an embryonic-like state. Direct reprogramming of somatic cells to a pluripotent state was accomplished in 2006, when Takahashi and Yamanaka (2006) converted adult mouse fibroblasts to iPSCs through ectopic expression of a selected group of transcription factors (i.e. Oct4, Sox2, Klf4 and c-Myc). Subsequent reports optimized this technique and direct reprogramming was achieved in human cells on feeder cells (Takahashi *et al*., 2007; Yu *et al*., 2007; Aasen *et al*., 2008; Dimos *et al*., 2008; Huangfu *et al*., 2008; Lowry *et al*., 2008; Maherali *et al*., 2008; Nakagawa *et al*., 2008; Park *et al*., 2008a, b; Ebert *et al*., 2009; Li *et al*., 2009), providing an invaluable contribution to the field of regenerative medicine. Realization of the promise of iPSCs will require improved methods of directed differentiation for generating homogenous populations of lineage-specific cell types, as well as elimination of the risks and drawbacks associated with the current iPSC protocol, including genetic manipulation, and the low-efficiency/slow kinetics of induction. Recent advances in using various genetic approaches have addressed some of the challenges associated with iPSC production. 'Safe'

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hiPSCs have been produced using a range of techniques: Cre-excisable viruses (Soldner *et al*., 2009), an oriP/EBNA1-based episomal expression system (Yu *et al*., 2009), small molecules (Li *et al*., 2009) and protein transduction (Kim *et al*., 2009). hiPSCs and hESCs are similar but not identical. They are similar in (i) all morphological attributes, including unlimited self-renewal; (ii) expression of key pluripotency genes; and (iii) proof of functional differentiation. However, hiPSCs do differ from hESCs; DNA microarray analyses detected significant differences between the two pluripotent stem cell lines (Takahashi *et al*., 2007).

One problem in the development of hiPSC and hESC culture is that these cells are vulnerable to apoptosis upon cellular detachment and dissociation. Future applications involving hiPSC- and hESC-derived cells or tissues depend upon establishment of an efficient cryopreservation protocol, to facilitate their storage and transportation. Current freezing approaches of hESCs involve slow-freezing (Fujioka *et al*., 2004; Ji *et al*., 2004; Heng *et al*., 2005; Ware *et al*., 2005; Heng *et al*., 2006, 2007; Li *et al*., 2008b, c; Martin-Ibanez *et al*., 2008b) and vitrification (Reubinoff *et al*., 2001; Richards *et al*., 2004; Zhou *et al*., 2004; Li *et al*., 2008a) protocols. However, these methods have an extremely poor survival rate of hESCs, are labor intensive and unsuited for handling bulk quantities of cells. In an effort to circumvent the problem of apoptosis in hESC culture, Watanabe *et al*. (2007) showed that addition of a selective Rho-associated kinase (ROCK) inhibitor, Y-27632, to the hESC medium increased colony formation of dissociated hESCs. More recently, Claassen *et al*. (2009) determined that Y-27632 significantly improves the recovery of cryopreserved hiPSCs and their growth upon subculture. However, many questions about ROCK inhibition in hESCs and hiPSCs remain. In this study, we address three questions. Can ROCK inhibition prior to freezing enhance the recovery of single dissociated hESCs and hiPSCs from frozen stocks? Can ROCK inhibition improve the recovery of cryopreserved single dissociated hESCs and hiPSCs when cultured directly in a serum-free medium in the absence of a growth-inactivated feeder layer? Can ROCK inhibition increase the cloning efficiency of frozen/thawed single dissociated hESCs and hiPSCs in serum- and feeder-free culture when it is added both in extracellular matrix (ECM)/Matrigel and medium, in comparison with conventional addition to medium only (Watanabe *et al*., 2007)?

Materials and Methods

hESCs and hiPSCs culture

The hESC lines, Royan H5 and Royan H6 (Baharvand *et al*., 2006), and hiPSC lines, Royan hiPSC1 and Royan hiPSC4, were used in these experiments. These lines were established under feeder-free culture from skin fibroblast by transduction of retroviral factors including Oct4, Sox2, c-Myc and Klf4 (Totonchi *et al*., 2009). The cells were passaged and maintained under feeder-free culture. Briefly, the cells were cultured in hESC medium containing DMEM/F12 medium (Gibco) supplemented with 20% knockout serum replacement (KOSR, Gibco), 2 mM L-glutamine (Gibco), 0.1 mM β-mercaptoethanol (Sigma), 1% non-essential amino acids (Gibco), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco), insulin–transferrin–selenite (Gibco) and 100 ng/ml basic-fibroblast growth factor (bFGF). The cells were grown in 5% CO_2 with 95% humidity and were passaged every 7 days. For passaging, the cells were washed once with phosphate-buffered saline (PBS; Gibco) and then incubated with DMEM/F12 containing a 1:1 ratio of collagenase IV (0.5 mg/ml, Gibco):dispase (1 mg/ml, Gibco), or collagenase IV (1 mg/ml) only at 37°C for 5–7 min. When colonies at the edge of the dish started dissociating from the bottom, the enzyme was removed and the plate washed with PBS. Cells were collected by gently pipetting and replated on Matrigel (Sigma)-coated dishes and the medium changed every other day.

Cryopreservation of hESCs and hiPSCs

For freezing, the hESCs and hiPSCs were dissociated as single cells by 0.05% Trypsin, 0.53 mM EDTA (Gibco) for 5 min at 37°C and then collected by gently pipetting. The ROCK inhibitor Y-27632 (Calbiochem, Cat. No. 688000) was added to the culture medium at a final concentration of 10 μM 1 h before detaching the cells. The single dissociated cells were frozen in freezing medium containing 10% DMSO (Sigma) plus (A) 90% FCS (Hyclone), or (B) 90% KOSR, or (C) 90% hESC culture medium (20% KOSR) which contained ROCK inhibitor Y-27632 (Martin-Ibanez *et al*., 2008a). $1-2 \times 10^6$ cells per 250 μl ice-cooled freezing medium were cryopreserved. This mixture was then cooled in a cell-freezing container and kept at −80°C overnight. The vials were transferred into liquid nitrogen tank next day for long-term storage.

Recovery of cryopreserved hESCs and hiPSCs

Cryopreserved stocks of hESCs and hiPSCs were recovered by partially submerging the vial of frozen cells in water at 37°C water bath until most (but not all) contents thawed, then transferred to a 15 ml conical tube with 4 ml complete hESC medium. The cells were centrifuged at 200*g* at room temperature for 5 min, and the supernatant discarded. The cells were resuspended in 3 ml of hESC medium containing 10 μM Y-27632. The cell suspension was transferred into a Matrigel-coated 60-mm dish in hESC medium containing 10 μM Y-27632 and incubated at 37°C, 5% CO_2 until the next day, when the medium was replaced with fresh medium without Y-27632.

Assessment of cell viability after cryopreservation

We measured the survival of the cells cryopreserved in the different groups by counting the number of live cells after freezing by using a hemocytometer. Cell viability was assessed by the Trypan Blue exclusion method (Anjomshoa *et al*., 2009).

Karyotype analysis

For karyotype analysis, the cells were treated with 0.66 μM thymidin (Sigma) for 16 h at 37°C in 5% CO_2. After washing, the cells were left for 5 h and then treated with colcemid (Gibco, 0.15 μg/ml, 30 min). Isolated hiPSCs and hESCs were exposed to 0.075 M KCl at 37°C for 16 min and then were fixed in three consecutive immersions in ice-cold 3:1 methanol:glacial acetic acid and then dropped onto pre-cleaned chilled slides. Chromosomes were visualized using standard G-band staining. At least 20 metaphase spreads were screened and 10 of them were evaluated for chromosomal re-arrangements.

Immunoflourescence and alkaline phosphatase staining

The cells were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 30 min and blocked in 10% goat serum in PBS for 1 h. Cells were incubated with primary antibody for 1 h at 37°C, washed and incubated with FITC-conjugated secondary antibodies,

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anti-mouse IgM (1:100, Sigma, F9259), anti-rat IgG (1:100, Sigma, F1763) and anti-mouse IgG (1:200, Sigma, F9006) as appropriate for 1 h at 37°C.

Primary antibodies were anti TRA-1-60 (1:100, Chemicon MAB4360), TRA-1-81 (1:100, Chemicon MAB4381), Oct4 (1:100, Santa Cruz Biotechnology, SC-5279), SSEA-3 (1:100, Chemicon, MAB4303) and SSEA-4 (1:100, Chemicon, MAB4304) for undifferentiated hESC determination and anti-Pax6 (1:200, Santa Cruz, SC-11357), Nestin (1:100, Chemicon, MAB5326), neuron-specific tubulin-III (1:250, Sigma, T5293) and microtubule-associated protein (MAP2; 1:200, Sigma; M1406) for neural cell detection. Nuclei were counterstained with propidium iodide (PI, Sigma) in some of the experiments. The cells were analyzed with a fluorescent microscope (Olympus, Japan).

Alkaline phosphatase (ALP) staining was performed on the basis of manufacturer's recommendations (Sigma).

RNA isolation and quantitative RT–PCR

Total RNA was isolated using the Nucleospin Kit (MN) and treated with DNaseI, RNase Free Kit (Fermentas) to remove genomic DNA contamination. Two micrograms of total RNA was used for reverse transcription reaction with the RevertAid First Strand cDNA Synthesis Kit (Fermentas) and random hexamer primer, according to the manufacturer's instructions. Quantitative PCR reactions were set up in duplicate with the Power SYBR Green Master Mix (Applied Biosystems) and analyzed with the 7500 real-time PCR system (Applied Biosystems). Expression values were normalized to the average expression of housekeeping gene (GAPDH). The sequences of primers for endogenous genes have been reported previously (Totonchi *et al*., 2009).

In vitro differentiation

To demonstrate whether frozen/thawed hESC and hiPSC clones produced under these conditions are pluripotent, they were assayed for their ability to differentiate into lineages representative of the three embryonic germ layers. Using standard protocols used for pluripotent stem cell differentiation, the hESCs and hiPSCs were subjected to the embryoid body (EB) formation assay. For EB formation, the cells were harvested by treating with collagenase IV/dispase. Clumps of cells (500–1000 cells) were transferred to bacterial or agarose-coated dishes to suspension culture in DMEM/F12 containing 20% fetal bovine serum, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% non-essential amino acids, penicillin and streptomycin. The medium was changed every other day. After 12 days, as a floating culture, EBs were transferred to a 0.1% gelatin-coated plate and cultured in the same medium for another 8 days. hiPSC1 and Royan H5 were differentiated to the neural lineage in defined adherent culture by retinoic acid, noggin and bFGF (Baharvand *et al*., 2007).

Colony formation after thawing of hESCs and hiPSCs

To evaluate the cloning efficiency of frozen/thawed hESCs and hiPSCs [(number of ALP-positive colonies/number of seeded cells) × 100] and assess ROCK inhibitor Y-27632 effect on it, we analyzed the number of feeder-independent colonies of dissociated single pluripotent stem cells. For this purpose, we established five experimental conditions on the basis of the presence or absence of Y-27632 in Matrigel or culture medium after thawing of hESCs and hiPSCs. Matrigel as coating substratum in the absence of ROCK inhibitor was diluted in DMEM/F12 medium (group 1) or hESC medium (group 2). ROCK inhibitor Y-27632 (10 μM) was also added into medium only as a group that was previously described by Watanabe *et al*. (2007) (group 3). Moreover, Matrigel supplemented with ROCK inhibitor was used only in Matrigel (group 4) or Matrigel and medium (group 5).

Statistical analysis

All experiments were conducted in at least three independent cultures. Results were expressed as the mean ± SD. Statistical analysis of qRT–PCR, viability and comparison of cloning efficiency was done using the REST-MCS version 2 software and one-way ANOVA followed by Tukey's *post hoc* test. The cloning efficiency (based on the ratio of ALP-positive colonies formed per initially seeded hESCs and hiPSCs) was analyzed by imageJ version 1.4. The mean difference was considered significant at the $P < 0.05$ level.

Results

Cryopreservation and recovery of hESCs and hiPSCs

To develop an efficient cryopreservation protocol of pluripotent stem cells (i.e. hESCs and hiPSCs) in feeder-independent conditions, we studied the effects of ROCK inhibitor Y-27632 on the plating of two different hESC lines (Royan H5 and Royan H6) and two hiPSC lines (Royan hiPSC1 and Royan hiPSC4) by the slow-freezing and rapid-thawing method. First, to assess the best freezing medium, the cells (as single cells, by trypsin/EDTA) were frozen in freezing medium containing 10% DMSO plus (A) 90% FCS, or (B) 90% KOSR and/or (C) 90% hESC culture medium (20% KOSR) which contained ROCK inhibitor Y-27632 (Fig. 1). Analysis of the survival rate of cryopreserved cells in the different groups by the Trypan Blue exclusion method showed that exposure to ROCK inhibitor Y-27632 in freezing solution alone does not enhance, but significantly reduces the post-thaw survival rate of dissociated single hESCs and hiPSCs (Fig. 1, group C). More viable cells were observed in the presence of 90% FCS (Fig. 1, $P < 0.01$). Therefore, we continued our freezing by using the method of group A.

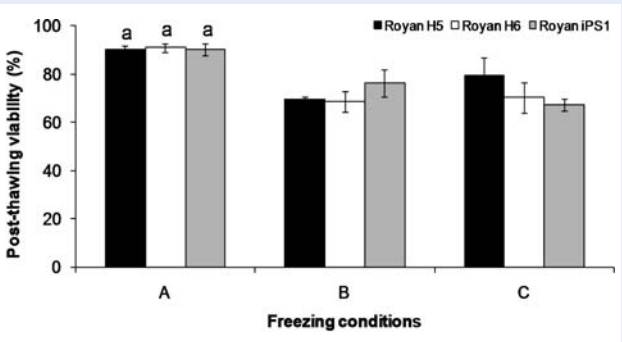

Figure 1 The survival rate of the pluripotent stem cells cryopreserved in freezing medium containing 10% DMSO plus 90% FCS (**A**), or 90% KOSR (**B**) or 90% hESC culture medium (20% KOSR) which contained ROCK inhibitor Y-27632 (**C**). The survival of the cells was measured by counting the number of live cells using a hemocytometer. Cell viability was assessed by the Trypan Blue exclusion method. The data presented are the mean percentage cell viability ± SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. a: $P < 0.01$, A versus B and C freezing conditions. DMSO, di-methyl sulfoxide; FCS, fetal calf serum; hESC, human embryonic stem cell.

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Assessment of pluripotency markers

After thawing, the cells were cultured in the presence of 10 μM ROCK inhibitor Y-27632 for 1 day. Seven days later, the colonies of hESCs and hiPSCs had clear borders (Fig. 2A) that consisted of compact cells with high nuclear:cytoplasm ratios (Fig. 2B). In addition, colonies were ALP-positive (Fig. 2C), and immunofluorescence staining showed that all colonies expressed the nuclear marker Oct4 (Fig. 2D), and the surface markers SSEA3 (Fig. 2E), SSEA4 (Fig. 2F), Tra-1-60 (Fig. 2G) and Tra-1-81 (Fig. 2H). To evaluate the percentage of undifferentiated cells, we analyzed the expression of Oct4, SSEA4 and Tra-1-60 or Tra-1-81 using two-color flow cytometry for hESC and hiPSC lines obtained before freezing and at passages P1 and P5 (for hiPSC1) and P1 and P10 (for hESC, Royan H5) after thawing (Fig. 3). Under these conditions, >80% of the cells expressed Oct4, SSEA4, Tra-1-60 and Tra-1-81. The hiPSC4 and Royan H6 also expressed the markers (data not shown). The cells were also double-positive for SSEA4/Tra-1-60 and SSEA4/Tra-1-81. Karyotype analysis was performed in hESCs and hiPSCs, five to eight passages after freeze/thaw, to demonstrate the absence of chromosomal abnormalities. The results showed normal karyotype for hESC and hiPSC lines (Fig. 2I and J).

The frozen–thawed dissociated hESCs maintained their undifferentiated state over time; no differences were found between the levels of pluripotency marker expression at any time point analyzed for either of the two hESC lines (Royan H5, Royan H6: data not shown) or hiPSC1 line when compared with undifferentiated control colonies, which were maintained in standard conditions (Fig. 4).

Finally, we studied the pluripotency of hESCs and hiPSCs *in vitro* after cryopreservation by examining EB formation at passages 5–14 post-thawing (Fig. 5). Tissue components expressing markers of the three germ layers, such as α-FP, FaxA2, Sox17 and Alb for endoderm, SOX1, Pax6 and Nestin for ectoderm and MEF2C, Brachyury, GATA4, TNNT2, NPPA and PPAR for mesoderm, were identified by RT–PCR (Fig. 5A) and immunocytochemistry for Pax6, Nestin, MAP2 and tubulin III (Fig. 5B).

Colony formation after thawing of hESCs and hiPSCs in the presence or absence of ROCK inhibitor in Matrigel and/or medium

Given the role of Y-27632 in cloning efficiency, we then analyzed the number of feeder-independent colonies of dissociated single pluripotent stem cells. Frozen–thawed hESCs and hiPSCs under different five conditions (see Materials and Methods) were seeded and allowed to grow for 7 days, after which the number of colonies was determined (Fig. 6). Matrigel was diluted in hESC medium in groups 3, 4 and 5. The cryopreservation of dissociated hESCs and hiPSCs was not achieved in the absence of ROCK inhibitor since very few colonies were obtained in groups 1 or 2, or the cloning efficiency was <1%. However, the addition of Y-27632 into medium (groups 3 and 5) significantly increased the number of colonies formed ($P < 0.001$). Moreover, the number of colonies formed after thawing showed that the presence of Y-27632 in the medium (group 3) was better than its presence in Matrigel only (group 4). Interestingly, the highest efficiency of colony formation was achieved by the addition

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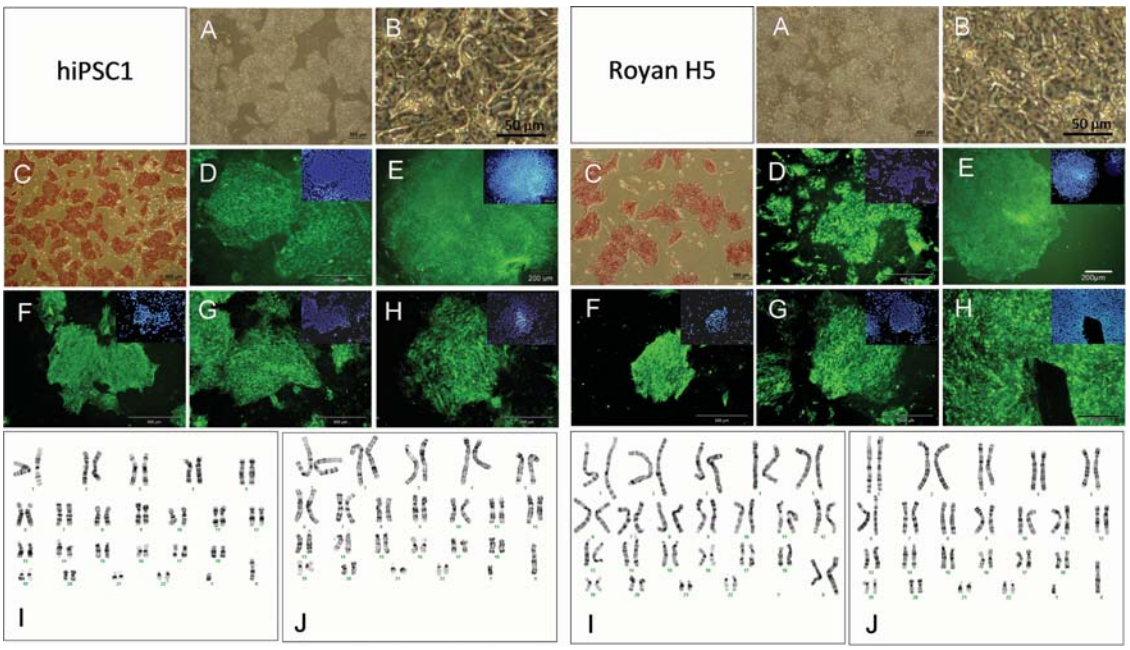

Figure 2 Characterization of pluripotency markers post-thaw hiPSC1 (left) and hESCs (Royan H5, right). (**A**) Morphology and (**B**) higher magnification of hiPSCs. Expression of (**C**) ALP, (**D**) Oct4, (**E**) SSEA3, (**F**) SSEA4, (**G**) TRA-1-60 and (**H**) TRA-1-81. The hiPSC4 and Royan H6 expressed the markers (not shown). The karyotype of hiPSC1 (**I**) and hiPSC4 (**J**) are normal. The karyotype of Royan H5 (I) and Royan H6 (J) and Royan H1 (not shown) was also normal. The lines are characterized after five to eight passages. Nuclei were stained with DAPI (blue). hESC, human embryonic stem cell; hiPSCs, human induced pluripotent stem cells.

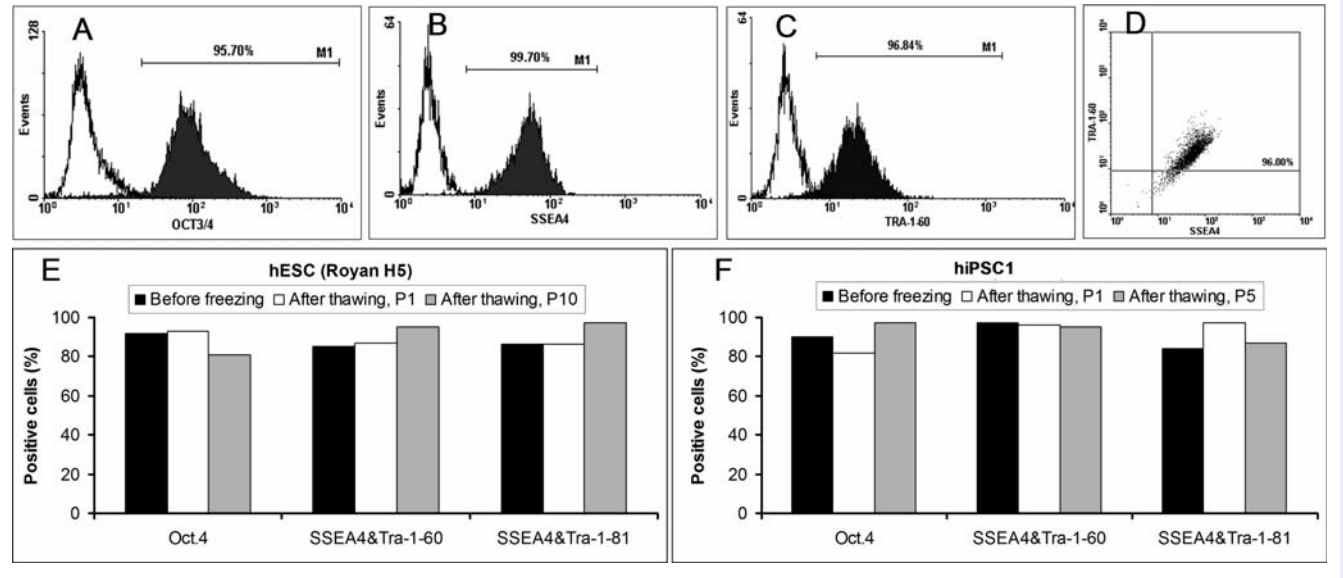

Figure 3 Expression of Oct4, SSEA4 and TRA-1-60 in hiPSCs and hESCs post-thaw in feeder-free condition is shown by flow cytometry. Representative samples of Oct4 (**A**), SSEA4 (**B**) and TRA-1-60 (**C**). The cells expressing antigen compared with isotype control (white peaks) were termed antigen-positive population. Percentages of double-positive for SSEA4 and TRA-1-60 are indicated in the dot plots (**D**). The percentages of positive cells indicated for hESCs (Royan H5, **E**) and hiPSC1 (**F**). hESC, human embryonic stem cell; hiPSCs, human induced pluripotent stem cells; P, passage.

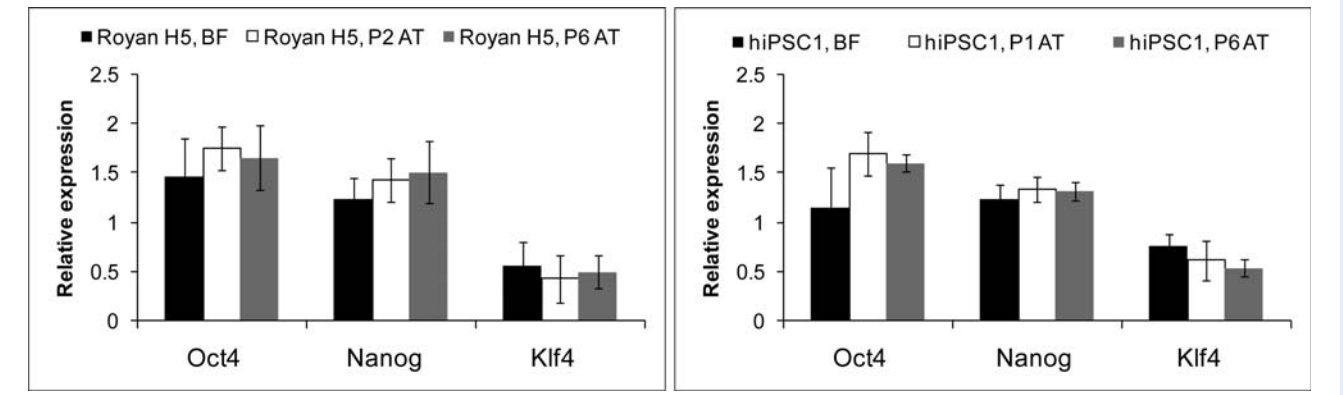

Figure 4 hESCs and hiPSCs maintained their undifferentiated state over time after single-cell cryopreservation in the presence of Y-27632. Real-time quantitative RT–PCR analysis shows no significant difference in the expression levels of pluripotency genes, Oct4, Nanog and Klf4 in hESCs (Royan H5, Royan H6: not shown) and hiPSC1 colonies obtained at passages (P), before freeze (BF), 2, and/or 6 after thawing (AT). Results are normalized to the levels of expression obtained from the average expression of housekeeping gene (GAPDH). Results are expressed as the mean of three independent experiments and error bars represent SD. Statistical analysis was performed using the REST-MCS. hESC, human embryonic stem cell; hiPSCs, human induced pluripotent stem cells.

of Y-27632 both in medium and Matrigel (group 5) post-thawing, which thereby had an additive effect (Fig 6, $P < 0.05$).

Discussion

Feeder-free propagation of hESCs (Amit and Itskovitz-Eldor, 2006; Ellerstrom *et al.*, 2006; Furue *et al.*, 2008) and more recently hiPSCs (Totonchi *et al.*, 2009) with Matrigel-coated plates has been reported. However, all the reviewed cryopreservation methods on hESCs show a low cloning efficiency in feeder-free culture, which is one of the important problems in storage and transportation of these cells.

Despite several attempts toward improving quantity and quality, there are few successes in the development of freezing and maintenance protocols for pluripotent feeder-free hESCs (Skottman *et al.*, 2007; Unger *et al.*, 2008) and no reports on feeder-free hiPSCs.

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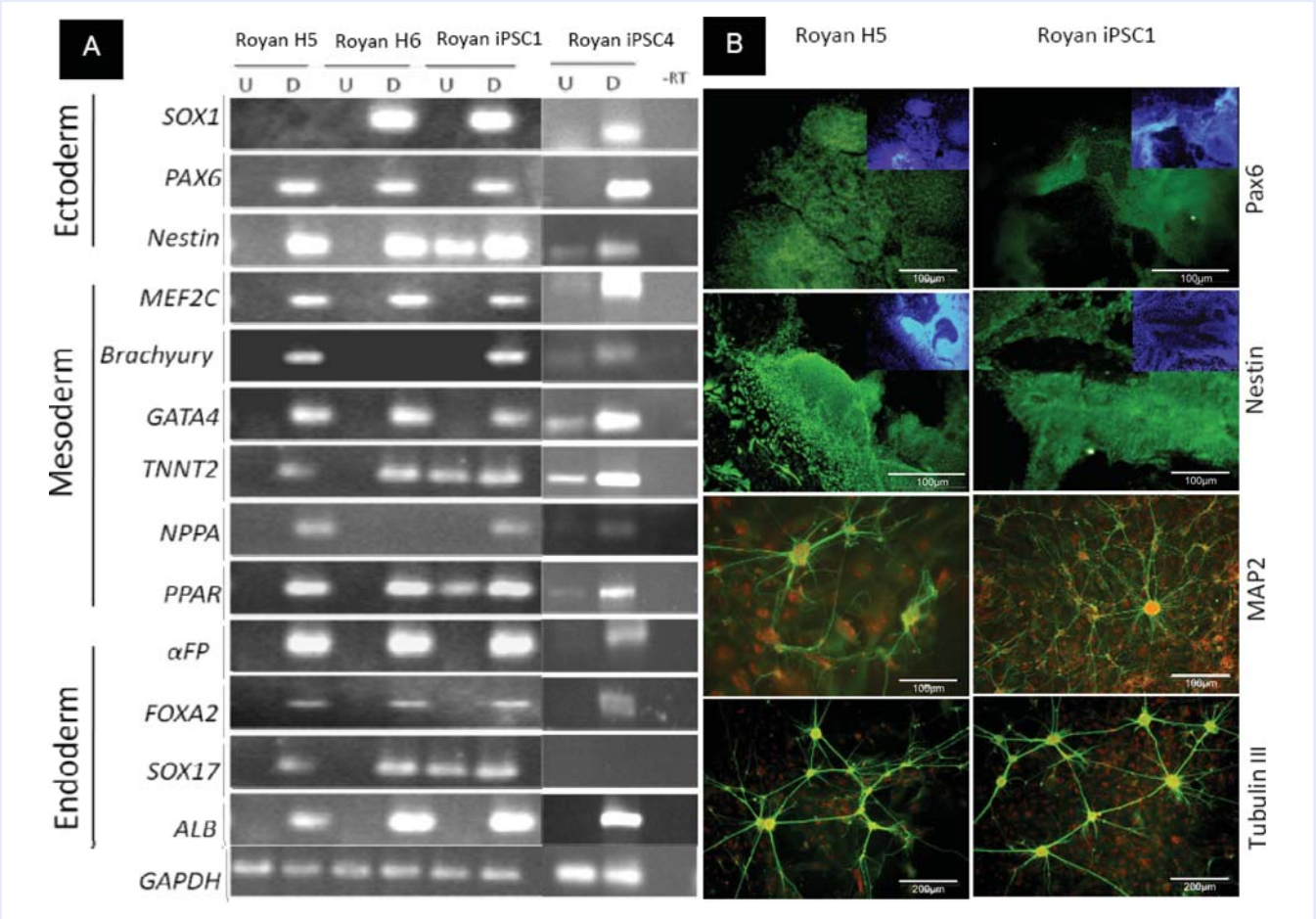

Figure 5 Cryopreserved dissociated hiPSCs and hESCs retained key properties of pluripotent markers. *In vitro* differentiation of hiPSCs and hESCs by EB formation at passage 6 after cryopreservation. (**A**) RT–PCR analysis of the expression of markers of the three embryonic germ layers. (**B**) Representative photomicrographs showing differentiated EBs expressing Pax6, Nestin, MAP2 and β-tubulin III markers. DAPI (blue color) or propidium iodide (red color) counterstaining is shown for nuclei. hESC, human embryonic stem cell; hiPSCs, human induced pluripotent stem cells; U, undifferentiated; D, differentiated.

Here, we report the effective freezing/thawing of single dissociated hESCs and hiPSCs in a feeder-free culture in the presence of ROCK inhibitor Y-27632. The presence of Y-27632 in both ECM and medium improved the efficiency of colony formation after thawing in comparison with the group that has Y-27632 in the medium only (Watanabe *et al*., 2007). No obvious adverse effects of continuous Y-27632 treatment on pluripotency or chromosomal stability in culture were observed, even after a substantial number of passages, confirming previous reports (Watanabe *et al*., 2007). ROCK inhibitors such as Y-27632 and Fasudil are already used clinically in cardiovascular therapies (Hu and Lee, 2005), suggesting that they are safe for use with these pluripotent stem cells.

ROCK inhibitors show anti-apoptotic activity and permit the survival of frozen/thaw dissociated single hESCs (Heng *et al*., 2006; Shi and Wei, 2007; Watanabe *et al*., 2007; Koyanagi *et al*., 2008; Li *et al*., 2008b, c; Lingor *et al*., 2008; Martin-Ibanez *et al*., 2008b) or monkey ESCs (Takehara *et al*., 2008) and more recently hiPSCs (Claassen *et al*., 2009). Heng *et al*. (2007) investigated whether a synthetic broad-spectrum irreversible inhibitor of caspase enzymes involved in apoptosis, Z-VAD-FMK, could be used to enhance the post-thaw survival rate of hESCs. Exposure to 100 mM Z-VAD-FMK in the freezing solution alone did not significantly enhance the post-thaw survival rate. However, when 100 mM Z-VAD-FMK was added to the post-thaw culture media, there was a significant enhancement in the survival rate, which was further increased when Z-VAD-FMK was also added to the freezing solution (Heng *et al*., 2007).

Recent studies have demonstrated that Y-27632 increased not only the survival rate but also the adhesion of frozen–thawed dissociated single pluripotent stem cells in the presence (Li *et al*., 2008c; Martin-Ibanez *et al*., 2008b; Claassen *et al*., 2009) and absence (Li *et al*., 2008b) of feeder cells. Li *et al*. (2008b) proposed that Y-27632 does not block apoptotic pathways, but rather causes the cells to become 'blind', 'deaf' and 'dumb' in respect to their current environment . Our real-time RT–PCR results showed that the expression of integrin chains αV, α6 and β1 increased significantly in the presence of ROCK inhibitor. Integrins are heterodimeric transmembrane molecules with large extracellular domains and relatively small cytoplasmic domains formed by α and β subunits that can

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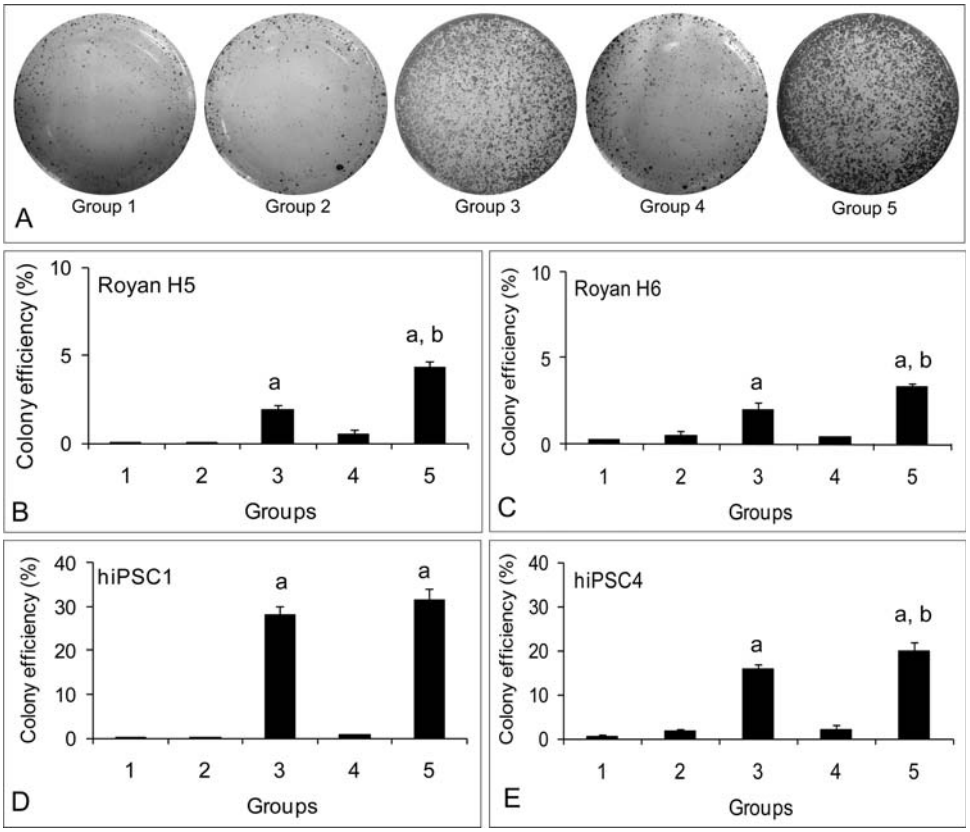

Figure 6 Number of colonies obtained after thawing of dissociated cryopreserved hiPSCs and hESCs in the five conditions established on the basis of the presence or absence of Y-27632 in the medium, Matrigel, or both. Group 1 and group 2 are controls, and in the absence of ROCK inhibitor, Matrigel was diluted in DMEM/F12 medium (group 1) or hESC medium (group 2). ROCK inhibitor Y-27632 (10 μM) was also added into medium only as a group that was previously described by Watanabe *et al.* (2007) (group 3). Moreover, Matrigel supplemented with ROCK inhibitor was used only in Matrigel (group 4) or Matrigel and medium (group 5). (**A**) A representative photograph of ALP-positive staining in different groups for hiPSC4. (**B**) Evaluation of cloning efficiency for hESC lines (Royan H5 and Royan H6) and two hiPSC lines (hiPSC1 and hiPSC4). Results are expressed as the mean of three independent experiments, and error bars represent the SD. The cloning efficiency was analyzed on the basis of the ratio of ALP-positive colonies formed per initially seeded hESCs and hiPSCs by ImageJ software. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. a: $P < 0.001$, 1, 2 and 4 versus 3 and 5; b: $P < 0.05$, 3 versus 5. ALP, alkaline phosphotase; hESC, human embryonic stem cell; hiPSCs, human induced pluripotent stem cells.

switch between inactive and active conformations. Moreover, the presence of ROCK inhibitor in Matrigel increases integrin expression and this may augment an undifferentiated state and cause adhesion of human ESCs and iPSCs to Matrigel and thereby allowing better cloning efficiency (unpublished work). This hypothesis is supported when Y-27632 suspension aggregates, normally observed after cryopreservation, can be dissociated with the addition of EGTA, a calcium chelator, and gentle pipetting (Li *et al.*, 2008b). The potential of Y-27632 to enhance cellular adhesion is also observed in other cell types; for example, the adhesion of human trabecular meshwork cells to fibronectin or collagen type I is increased by the addition of Y-27632 (Koga *et al.*, 2006). Y-27632 is a selective inhibitor of Rho kinase. Rho accepts signals from G-protein-coupled receptors in addition to other signaling pathways that originate in the ECM, as well as intracellularly (Leung *et al.*, 1996; Amano *et al.*, 1997; Ishizaki *et al.*, 1997; Kawaguchi *et al.*, 2000; Honjo *et al.*, 2001). Rho activation of ROCK leads to the phosphorylation of a number of downstream targets which are involved in diverse signaling pathways. Intriguing recent findings also suggest significant potential for ROCK inhibitors in the production and implantation of stem cells for disease therapies (for review, see Olson, 2008). 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. Moreover, recent papers by Peerani *et al.* (2007) and Harb *et al.* (2008) have brought to light the role of ROCK within the maintenance of hESC pluripotency. Peerani *et al.* (2007) demonstrated that Y-27632 treatment increased levels of Oct4 expression, and Harb *et al.* (2008) demonstrated that hESCs can be grown without the need for niche-forming feeder layers or animal-derived matrices with the addition of Y-27632 in a single synthetic matrix, i.e. poly-D-lysin.

Our protocol uses trypsin/EDTA to dissociate single hESCs and hiPSCs. Conventionally, hESCs are passaged as clusters by collagenase/dispase, or as dissociated single cells by trypsin. However, recently, Bajpai *et al.* (2008) demonstrated that hESCs can be passaged as single cells using Accutase, a formulated mixture of digestive enzymes. The advantages of Accutase over the traditional trypsin/

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EDTA treatment are that it is less damaging to cells, leading to increased viability, and carries a lower risk of introducing adventitious agents into cell cultures because it does not contain any mammalian or bacterially derived proteins. Therefore, investigation of the inclusion of Accutase in this method may lead to further increases in the cloning efficiency of hESCs and hiPSCs.

Taken together, these data demonstrate that the addition of Y-27632 to medium increases the survival of single dissociated hESCs and hiPSCs in feeder-free culture. The cloning efficiency of hiPSCs and hESCs improves when ROCK inhibitor is added both in Matrigel and in medium in comparison with conventional addition to medium alone. Addition of ROCK inhibitors such as Y-27632 to synthetic matrices, such as poly-D-lysine, its presence in culture medium and using of safer dissociated enzymes, such as Accutase, may bring us one step closer to attaining the quality and quantities of pluripotent stem cells that will be required for clinical purposes, once the safety issue is overcome.

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