**BACKGROUND:** Efficient slow freezing protocols within serum-free and feeder-free culture systems are crucial for the clinical application of human embryonic stem (hES) cells. Frequently, however, hES cells must be cryopreserved as clumps when using conventional slow freezing protocols, leading to lower survival rates during freeze-thaw and limiting their recovery and growth efficiency after thawing, as well as limiting downstream applications that require single cell suspensions. We describe a novel method to increase freeze-thaw survival and proliferation rate of single hES cells in serum-free and feeder-free culture conditions.

**METHODS:** hES cells maintained on Matrigel-coated dishes were dissociated into single cells with Accutase and slow freezing. After thawing at 37°C, cells were cultured in mTeSR medium supplemented with 10 μM of Rho-associated kinase inhibitor Y-27632 for 1 day.

**RESULTS:** The use of Y-27632 and Accutase significantly increases the survival of single hES cells after thawing compared with a control group (P<0.01). Furthermore, by treatment of hES cell aggregates with EGTA to disrupt cell-cell interaction, we show that Y-27632 treatment does not directly affect hES cell apoptosis. Even in the presence of Y-27632, hES cells deficient in cell-cell interaction undergo apoptosis. Y-27632-treated freeze-thawed hES cells retain 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:** The method described here allows for cryopreservation of single hES cells in serum-free and feeder-free conditions and therefore we believe this method will be ideal for current and future hES cell applications that are targeted towards a therapeutic end-point.

**Key words:** human embryonic stem cells / Rho-associated kinase inhibitor Y-27632 / cryopreservation / apoptosis

Human embryonic stem (hES) cells are derived from the inner cell mass (ICM) of human blastocysts. They have the potential for unlimited expansion with retention of normal karyotype and the ability to differentiate into multi-lineage cell types from all three germ layers (Thomson et al., 1998; Reubinoff et al., 2000). These properties make hES cells an attractive cell source for a variety of tissue regeneration applications, and therefore may offer a potential treatment alternative for many non-curable degenerative diseases and injuries.

Successful clinical implementation of hES cells-based technologies will rely on the development of an efficient cryopreservation method that utilizes serum-free and feeder-free culture conditions. Current methods involve slow or fast freezing (vitrification) protocols in the presence of cryoprotectants [dimethylsulphoxide (DMSO), polyols, etc]. Vitrification (fast freeze, slow thaw) is extremely labor-intensive and unsuited for handling bulk quantities of hES cells, whereas conventional slow freezing (fast thaw) protocols have not proven to be efficient for hES cells. Here, the typical survival rate is c.10% (Reubinoff et al., 2001; Fujioka et al., 2004; Richards et al., 2004; Zhou et al., 2004; Heng et al., 2006).

All current hES cell protocols rely on cryopreserving small clumps of cells to improve survival rate as hES cells do not passage well as single cells. However, there are complications to freezing clumps, one being limitations on cryoprotectant exposure inside the clump. Indeed, only a handful of cells in the clump may survive cryopreservation, but remain attached to dead cells, causing a broader cell death response. The use of hES cell clumps also prevents a good estimation of freezing/passageing efficiency, as precise cell numbers are never truly known. Additionally, the passing of hES cells as clumps prevents other challenges for downstream manipulation. Examples include, cell transfection, cell separation by flow cytometry and high throughput screening (Ruchi et al., 2008).
In contrast to clumps of hES cells, single hES cells are more vulnerable to apoptosis upon cryopreservation (Watanabe et al., 2007). Using current published methods single hES cells have not been successfully cryopreserved, although many groups have reported low survival rates freezing hES cell clumps (Reubinoff et al., 2001; Fujikake et al., 2004; Richards et al., 2004; Zhou et al., 2004; Heng et al., 2006). Recently, some reports showed that the application of a selective Rho-associated kinase (ROCK) inhibitor, Y-27632 (Narumiya et al., 2000), and Accutase, a formulated mixture of digestive enzymes, to hES cell passaging permits survival of dissociated hES cells and markedly increases cloning efficiency (Watanabe et al., 2007; Koyanagi et al., 2008; Ruchi et al., 2008). In addition, many existing hES cell lines have been maintained using mouse embryonic fibroblast (MEF) feeder cells and animal serum medium. Although MEF and MEF-conditioned medium support hES cells self-renewal, the use of animal-derived components in hES cell culture precludes the use of these cells in clinical applications.

In the current study, we have demonstrated that Y-27632 treatment along with Accutase can be used to enhance the post-thaw survival rate of single hES cells using conventional slow freezing and rapid thawing protocols in serum-free and feeder-free culture conditions.

Materials and Methods

Cells and animals
The present study adheres to the guidelines for hES cell research established by the Government of Canada and the Canadian Institutes of Health Research. All experimental protocols and animal handling procedures were reviewed and approved by the Laboratory Animal Care and Use Committee of the University of Calgary.

Maintenance and passaging of hES cells
H9 hES cells (WiCell Research Institute) were maintained on MEF feeder cells and transferred onto Matrigel (BD Biosciences) -coated 35 mm dishes (Nunc) in mTeSR1 media (StemCell Technologies Inc.) following the manufacturer’s instructions. For passaging, hES cells were first washed twice with Dulbecco’s phosphate-buffered saline (DPBS) without calcium and without magnesium (Invitrogen) and then dissociated with Accutase (Chemicon) cell detachment solution in a 37°C incubator for 8 min. After gentle pipetting, the cells were collected by centrifugation and replated on Matrigel-coated dishes at different densities. On the first day after replating, the medium was supplemented with 10 μM of Y-27632. From the second day onward, the medium was changed daily without Y-27632 supplementation. The experimental design consisted of five freeze-thaw events that took place within 15 passages of the H9 cells in serum-free and feeder-free culture conditions. The analysis presented in this study, including karyotype, immunofluorescence, and in vitro and in vivo differentiation potential analysis, was carried out after 15 passages.

Cell adhesion assays
Cell adhesion assays were performed using the Vybrant Cell Adhesion Assay Kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer’s instructions with minor modifications. Briefly, single hES cells were added to a Matrigel-coated microwell at a density of 1 x 10^5 per well. After 12 h the media, with or without 10 μM of Y-27632, was replaced with fresh media containing 5 μM of calcein acetoxyethyl ester and incubated at 37°C for 30 min. Non-adherent cells were removed by gently washing twice with Dulbecco’s Modified Eagle’s Medium (DMEM)/F12 medium. Adherent cells were quantified using a fluorescence microplate reader (Fluoroskan Ascent, Thermo Labsystems) at 494 nm excitation and 517 nm emission. All experiments were repeated at least five times. Statistical analysis was performed using the Student’s t-test. Values of P < 0.05 were considered significant.

Karyotype analysis
Karyotype analysis of hES cells was carried out using the G-banding method. Briefly, cells were incubated with 0.05 μg/ml of colcemid at 37°C for 35 min, trypsinized, resuspended and incubated in 0.56% KCl for 25 min at 37°C, then fixed with 3:1 methanol/glacial acetic acid three times and then dropped onto slides to spread the chromosomes. The slides were baked overnight at 55°C, then treated with 0.05% trypsin for 90 s and stained with Giemsa and Leishman’s solution.

Immunofluorescence
The characterization of hES cells was carried out by measuring the expression of specific pluripotent markers including, alkaline phosphatase (AKP), stage-specific embryonic antigen (SSEA)-3, SSEA-4, tumor rejection antigens (TRA)-1-60, TRA-1-81 and octamer 4 (OCT-4). The immunostaining was done according to published procedures. Briefly, colonies were fixed in 4% paraformaldehyde for 30 min at room temperature, permeabilized with 0.1% Triton-X 100 in DPBS for 15 min at room temperature, washed three times with DPBS and incubated with DPBS containing 10% normal goat serum for 30 min at room temperature. Primary antibody (1:100) was applied at 4°C overnight. The negative control consisted of mouse immunoglobulin (IgG)2B (Caltag, Burlingame, CA, USA) at the same concentration as the primary antibody. After washing three times with DPBS, cells were incubated with either FITC-conjugated goat anti-mouse IgG (H+L) (1:100, Jackson ImmunoResearch Labs) or Alexa
Fluoro® 546 goat anti-mouse IgG (H+L) (1:300, Invitrogen). Histochemical staining for AKP was carried out using a commercially available kit following the manufacturer’s instructions (Chemicon).

**In vitro and in vivo differentiation**

Multilayer hES cell clumps were aggregated using 35 mm agar-coated dishes (Nunc) to produce embryoid bodies (EBs) in suspension culture which were then cultured in differentiation medium. The differentiation medium consisted of 80% DMEM (Invitrogen), 20% fetal bovine serum (Invitrogen), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol and 0.1 mM non-essential amino acids. After 7–14 days, the cells aggregated and generated cystic EBs. RT–PCR analysis on undifferentiated cells, Day 7 and Day 14 EBs were performed by using primers specific for each germ layer as previously described (Yoo et al., 2005), namely, ectoderm (neurofilament-68 and keratin), mesoderm (enolase and kallikrein) and endoderm (α-fetoprotein and α-antitrypsin). Also, the EBs of Day 14 were transferred onto gelatin-coated 4-well plates (Nunc) and cultured for an additional 4 days. The resulting cell types were analyzed by immunofluorescence using monoclonal antibodies against ectoderm (β-III tubulin III, Sigma, 1:1000), mesoderm (smooth muscle actin, Sigma, 1:1000) and endoderm (α-fetoprotein, Sigma, 1:1000) as previously described (Li et al., 2005). In addition, cells were injected into severe combined immunodeficient-beige mice (5 x 10³, Sigma, 1:1000), mesoderm (smooth muscle actin, Sigma, 1:1000) and endoderm (α-fetoprotein and α-antitrypsin). Also, the EBs of Day 14 were transferred onto gelatin-coated 4-well plates (Nunc) and cultured for an additional 4 days. The resulting cell types were analyzed by immunofluorescence using monoclonal antibodies against ectoderm (β-III tubulin III, Sigma, 1:1000), mesoderm (smooth muscle actin, Sigma, 1:1000) and endoderm (α-fetoprotein, Sigma, 1:1000) as previously described (Li et al., 2005). In addition, cells were injected into severe combined immunodeficient-beige mice (5 x 10³ cells per injection site) in order to examine the in vivo differentiation potential of the freeze-thawed hES cells. Teratomas were removed after 6 weeks, fixed overnight in 4% paraformaldehyde, embedded in paraffin, sectioned and examined histologically after being stained with eosin and haematoxylin.

**Influence of EGTA on hES cells treated with the rock inhibitor Y-27632**

EGTA was added into mTeSR medium to chelate extracellular calcium ions. The hES colonies were dissociated into single cells by Accutase and replated on Matrigel-coated and agar-coated dishes at a density of 1 x 10⁶ cells per 35 mm dish. Agar-coated dishes were prepared as follows: a 1% agar solution was autoclaved and allowed to slightly cool at room temperature, then applied to culture dishes to completely coat the bottom of the dish with the excess being aspirated immediately. The thin coat of agar was allowed to solidify and was then ready for use. The media were supplemented with different concentrations of EGTA (1 mM; 2 mM; 5 mM) and 10 µM of Y-27632. Each treatment group was cultured for 24 h at 37°C and the cell attachment in Matrigel-coated dishes, and cell aggregation in agar-coated dishes, was observed.

**Results**

**ROCK inhibitor Y-27632 increases adherent properties of hES cells**

When hES cells are cultured for 1 h in the presence of 10 µM of Y-27632 on Matrigel-coated dishes, we found that hES cells become more adherent, as it required more time in the presence of Accutase to detach cells from the culture dishes. When the cells reached 80% confluence and required passing, they normally require 8 min of Accutase treatment at 37°C to completely dissociate into single cells. However, cells treated with Y-27632 required 16 min of Accutase treatment at 37°C for complete dissociation. To elucidate the mechanism behind this observation, hES cells were treated with EGTA to chelate calcium in the presence or absence of Y-27632 to disrupt cadherin activity. As a note, mTeSR medium contains 1 mM calcium, and as one molecule of EGTA binds four calcium ions, 1 mM EGTA should be sufficient to sequester most extracellular calcium. After 24 h on Matrigel-coated dishes, cells attached and formed monolayer colonies with Y-27632 treatment (Fig. 2A). The addition of 1 mM EGTA did not affect the cell to dish attachment, however, the attached cells were far more dispersed than the Y-27632 treatment group alone (Fig. 2B). As expected, EGTA-treated cells displayed reduced cell–cell attachment and hES cells had a reduced colony formation capacity compared with control groups. When EGTA was removed from medium, the effect was reversed and cells adhered to each other with the colonies becoming tight and compact. However, fewer cells attached with 2 mM (Fig. 2C) and 5 mM (data not shown) of EGTA supplement. At higher concentrations of EGTA we can assume that intercellular calcium levels are severely affected.

To further study the effect of cell–cell attachment on cell survival, we cultured post-thawed cells in suspension culture prior to replating. When single hES cells are plated in agar-coated dishes, the cells do not adhere and will normally undergo apoptosis. However, in the presence of Y-27632, the hES cells aggregate into small clumps which are viable. When post-thawed single hES cells were cultured in suspension for 24 h on agar-coated dishes, no viable cells remained (Fig. 2D). However, when the medium was supplemented with...
small aggregates formed and viability increased (Fig. 2E). The aggregates were tight, spherical, smooth, and were difficult to dissociate into single cells by Accutase. When EGTA 1 mM was added into medium with Y-27632, aggregates were present, but they did not form a compact structure, with individual cells clearly visible within each aggregate (Fig. 2F, inset). The aggregates formed in the presence of EGTA required only gentle pipetting to dissociate and most importantly produced no viable colonies when replated onto Matrigel-coated dishes. Because of recent reports concerning the ROCK inhibitor and reduction of apoptosis (Watanabe et al., 2007; Koyanagi et al., 2008), our observation prompted us to explore the effect of Y-27632 on apoptosis seen during the freeze-thaw of hES cells.

Figure 1  The attachment and morphology of freeze-thawed and regularly passaged H9: human embryonic stem (hES) cells at different replating densities with or without Y-27632 treatment. The plating densities used were 1 × 10^5 cells/dish (A, E, I and M), 3 × 10^5 cells/dish (B, F, J and N), 5 × 10^5 cells/dish with Y-27632, the Rho-associated kinase (ROCK) inhibitor, treatment (C, G, K and O) and 5 × 10^5 cells/dish without Y-27632 treatment (D, H, L and P). Freeze-thawed cells, were froze (one week) as single cells, rapidly thawed, and seeded on Matrigel-coated 35 mm dishes. Pasaged control cells were dissociated into single cells and immediately plated on Matrigel-coated 35 mm dishes. Day 1 and Day 3 represent days after replating in cryopreserved and passaged H9 cells. Scale bar indicates 100 μm.

10 μM of Y-27632, small aggregates formed and viability increased (Fig. 2E). The aggregates were tight, spherical, smooth, and were difficult to dissociate into single cells by Accutase. When EGTA 1 mM was added into medium with Y-27632, aggregates were present, but they did not form a compact structure, with individual cells clearly visible within each aggregate (Fig. 2F, inset). The aggregates formed in the presence of EGTA required only gentle pipetting to dissociate and most importantly produced no viable colonies when replated onto Matrigel-coated dishes. Because of recent reports concerning the ROCK inhibitor and reduction of apoptosis (Watanabe et al., 2007; Koyanagi et al., 2008), our observation prompted us to explore the effect of Y-27632 on apoptosis seen during the freeze-thaw of hES cells.

ROCK inhibitor protects single hES cells from apoptosis after cryopreservation

Freeze-thawed single hES cells treated with or without Y-27632 were cultured for 24 h on Matrigel-coated and agar-coated dishes and subjected to apoptosis analysis using Annexin V-FITC Apoptosis Detection Kit (Fig. 3). The apoptosis analysis was repeated at least three times and demonstrated consistent results. In the passaged control cells that were not cryopreserved, 80.34% cells were viable (both Annexin V-FITC and PI negative), 5.90% cells were in early apoptosis (Annexin V-FITC-positive and PI-negative) and 11.07% cells were in late apoptosis or dead (both Annexin V-FITC and PI-positive; Fig. 3A). This is in contrast to hES cells that had been freeze-thawed without ROCK inhibitor and not cultured after freezing prior to analysis, where only 65.53% cells were viable, 15.70% cells were in early apoptosis and 16.80% cells were in late apoptosis or dead (Fig. 3B).

We next tested the effect of ROCK inhibitor on apoptosis when cells where cultured in agar-coated dishes for 24 h after freeze-thaw before analysis. When hES cells were cultured without ROCK inhibitor in suspension for 24 h, 0.72% cells were viable, 5.81% cells were in early apoptosis and 92.54% cells were dead (Fig. 3C). However, when the suspension culture was supplemented with 10 μM of Y-27632, cells adhered together and formed compact aggregates (Fig. 2E). The aggregates were dissociated into single cells by Accutase and immediately subjected to apoptosis analysis. Data showed that
53.03% cells were viable, 14.76% cells were in early apoptosis and 27.29% cells were dead (Fig. 3D). Statistical analysis demonstrated that Group A versus Group B, and Group C versus Group D, were significant \((P < 0.01)\). Therefore, more hES cells were viable with Y-27632 treatment compared with the non-cryopreserved or freeze-thaw groups without Y-27632 treatment. Y-27632 significantly decreased apoptosis and enhanced the post-thaw survival rate of cryopreserved single hES cells.

Pluripotency of cryopreserved and passaged single hES cells

In order to confirm that the hES cells maintain pluripotent characteristics after slow freezing as single cells in serum-free and feeder-free culture conditions, we examined six key pluripotent markers, karyotype, and the in vitro and in vivo differentiation potential of hES cells after five rounds of freeze-thaw. hES cells maintained their characteristic undifferentiated morphology, and immunofluorescence staining for the key markers of pluripotent hES cells (AKP, OCT-4, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81) confirmed that cells within hES cell colonies were positive for these markers (Fig. 4). The undifferentiated cells proliferated and formed characteristic undifferentiated hES cell colonies when they were replated on Mitomycin C-treated MEF feeder layer (Fig. 5A).

For in vitro differentiation examination, the hES cells were re-cultured on MEF feeder layers to form multilayer colonies. These colonies were cut into small clumps and cultured in agar-coated dishes. After 7–14 days in suspension, the cells aggregated and generated cystic EBs (Fig. 5B). RT–PCR analysis was performed on RNA extracted from undifferentiated cells, Day 7 EBs and Day 14 EBs (Fig. 5C). The mRNA levels for specific genes for three germ layers (neurofilament-68 for ectoderm, enolase and kallikrein for mesoderm, \(\alpha\)-fetoprotein and \(\alpha\)-1-antitrypsin for endoderm) increased during differentiation (Fig. 5C). The EBs from Day 14 were transferred onto gelatin-coated 4-well plates and cultured for an additional 4 days. At this point, the EBs were positively stained for \(\beta\)-tubulin III (ectoderm), smooth muscle actin (mesoderm) and \(\alpha\)-fetoprotein (endoderm) (Fig. 5D–F).

In order to examine the in vivo differentiation potential of the freeze-thawed hES cells, they were injected into severe combined immunodeficient-beige mice and teratomas were removed after 6 weeks and examined histologically. Rosettes of neural epithelium...
(ectoderm, Fig. 6A), adipose cells (mesoderm, Fig. 6B) and gut-like epithelium (endoderm, Fig. 6C) were all observed indicating pluripotency of the injected cells. Karyotype analysis of hES cells was performed using G-banding method. These cells exhibited normal karyotypes and a representative karyotype from H9 cells is shown in Fig. 6D. Our data strongly suggest that hES cells maintain the characteristic morphology, normal karyotype and pluripotent potential after cryopreservation as single cells and long-term culture in serum-free and feeder-free culture conditions.

Discussion

Apoptosis is a regulated physiologic process which occurs during embryonic development and is seen within maintenance of tissue homeostasis and self renewal, where it plays very important roles in cell proliferation and differentiation. Embryonic stem cells removed from the developing ICM can be cultured in vitro under specific culture conditions, however, hES cells are very sensitive to their environment including medium composition, temperature, CO₂ levels, pH and attachment (Ludwig et al., 2006). When these environmental factors fall outside of a narrow window we observe dramatic cell death. Even during regular passaging of hES cells we can always observe significant cells in suspension and dead single cells. If regular maintenance of hES cells results in significant apoptosis and cell loss then it serves that cryopreservation, dissociation and mechanical manipulation induce significant apoptosis, and can render a culture non-viable (Katkov et al., 2006).

Recent reports have demonstrated that Y-27632 is a potent inhibitor of apoptosis and permits the survival of dissociated hES cells (Watanabe et al., 2007; Koyanagi et al., 2008). In these studies both groups determined that apoptosis of dissociated hES cells was markedly decreased with Y-27632 treatment in vitro and in vivo. 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 hES cell pluripotency. Peerani et al. (2007) demonstrated that Y-27632 treatment increased levels of Oct-4 expression, and Harb et al. (2008) demonstrated that hES cells can be grown without the need for niche-forming feeder layers or animal-derived matrices with the addition of Y-27632. Although, we do not directly demonstrate increased pluripotency with Y-27632 treatment, we do observe Oct-4 staining consistent with un-frozen controls in our study and decreased apoptosis with Y-27632 treatment.

In this study, after freeze-thawed cells were cultured in suspension for 24 h, we found dramatic differences in the level of apoptosis in cryopreserved cells with and without Y-27632 treatment (Fig. 3C and D). Our results demonstrate that suspension culture can induce cell apoptosis of hES cells and, consistent with previous reports (Watanabe et al., 2007; Koyanagi et al., 2008), we found that Y-27632 treatment decreased the level of apoptosis. When regularly passaged hES cells were dissociated into single cells and immediately subjected to apoptosis analysis, a small percentage of cells were dead and/or apoptotic (Fig. 3A), with this cell death most likely induced by Accutase dissociation. When these dissociated single cells were cryopreserved in liquid nitrogen (for 1 week), thawed and then immediately subjected to apoptosis analysis, we observed an increase in cell mortality (Fig. 3B), however, these results signify only a small percentage of cells died as a result of cryopreservation.

Other groups have demonstrated in mouse ES cells that Accutase dissociation and single cell suspension induces more cell death than that of cryopreservation alone in the recovery of freeze-thawed hES cells (Koyanagi et al., 2000). Moreover, this result by itself does not explain why we observed fewer viable cells after freeze-thaw, although most of the freeze-thawed cells were viable (Fig. 3B), and fewer cells can attach and form colonies when they were replated and cultured for 24 h (Fig. 1D). Whereas apoptosis is the major cause of the low survivability of hES cells after freezing-thawing (Heng et al., 2006), we believe that hES cells are sensitive to anokis (detachment induced apoptosis), and that the increased levels of apoptosis we and others have observed in suspension culture without Y-27632 treatment results from the loss of adherence to the substrate.
However, with Y-27632 treatment we do not see apoptosis in suspension culture after cryopreservation. In addition, Y-27632 treatment allows for stronger cell–cell interaction between hES cells, which is evident during Accutase dissociation, and aggregate formation in suspension culture (Fig. 2E).

Our data indicates that increased cellular adhesion induced by Y-27632 enhances the survival of single hES cells in feeder-free and serum-free culture conditions as well as single cell cryopreservation. The potential of Y-27632 to enhance cellular adhesion has also been observed in other cell types, for example, adhesion of human trabecular meshwork cells to fibronectin or collagen type I was increased by the addition of Y-27632 (Koga et al., 2006). Y-27632 is a selective inhibitor of ROCK (Rho kinase). Rho accepts signals from G-protein-coupled receptors as well as other signaling pathways that originate in the extracellular matrix (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.

To generalize one role of ROCK is that this protein transduces signals from the cortical actin cytoskeleton and ECM to the nucleus, leading to changes in cell morphology as well as transcriptional regulation. We hypothesize that by interrupting signals from the cellular environment by inhibiting ROCK, hES cells are no longer aware of their current environment. Therefore, hES cells treated with ROCK inhibitor are no longer able to detect that they have been dissociated
into single cells or the current state of the plasma membrane or actin cytoskeleton during/after cryopreservation. In effect we do not believe that Y-27632 blocks apoptotic pathways but more accurately causes hES cells to become ‘blind’, ‘deaf’ and ‘dumb’ in respect to their current environment, and this naivety allows hES cells to aggregate into small colonies and this cell–cell interaction is responsible for the reduction in apoptosis observed in Y-27632 treatment groups. This hypothesis is supported when Y-27632 suspension aggregates that are normally observed after cryopreservation can be dissociated with the addition of EGTA and gentle pipetting.

EGTA is a chelating agent that has been employed for many decades for sequestering free calcium within a micro-environment. Calcium, a co-factor for many proteins is essential for the proper function of classical cadherin molecules. Cadherins play an important role in cell and developmental biology, where they bind only to identical cadherin molecules bound within the plasma membrane of adjoining cells. These homophillic interactions are responsible for cell–cell interactions between cells/tissues expressing similar cadherins. In development, cadherin molecules are responsible for the correct patterning of many tissues and organs. With the addition of EGTA to Y-2732 treatment groups to chelate calcium, thereby inactivating cadherins, we observed no viable cells present after replating. This result signifies that Y-27632 does not block apoptosis in dissociated hES cells, but allows cells to aggregate and escape anokis. We recognize that more testing is required to support this hypothesis, however, our current and previous studies point in this direction. Aside from Y-27632 treatment, initial cell plating density affects proliferation of hES cells.

There are approximately $5 \times 10^6$ hES cells on a 90% confluent 35 mm dish. When the cells are dissociated, frozen, thawed and replated at a density of $1 \times 10^5$ cells per 35 mm dish, the split rate will be 1:50. These cells can be re-passaged after 5 days culture. This high proliferation efficiency demonstrates that hES cells have been successfully cryopreserved as compared with other cell lines using the standard slow freezing and rapid thawing protocols. The time of passaging depended on the initial plating density. At the same densities, the passaging time of freezing-thawing single cells was shortened by 0.5 days compared with regularly passaged hES cells.

**Figure 6** *In vivo* differentiation and karyotype of cryopreserved H9 cells. *In vivo* teratoma analysis of cryopreserved H9 cells demonstrates that the hES cells are capable of differentiating into tissue types derived from all three germ layers (A–C). Rosettes of neural epithelium (ectoderm, A), adipose (mesoderm, B); gut like epithelium (endoderm, C). Karyotype analysis of hES cells after cryopreservation shows that they have a normal chromosome complement of 46XX (D). Scale bar indicates 100 μm.
cells. Increasing cell plating density showed that more cells will attach, merge and then accelerate monolayer colony formation (Fig. 1). The formed monolayer colonies on Matrigel express pluripotent markers and maintain differentiation potential similar to multilayer colonies maintained on a MEF feeder layer (Li et al., 2008). No adverse effect of monolayer cultures on hES cell viability or pluripotency was detected, and is consistent with previous reports (Ruchi et al., 2008).

In addition to Y-27632 treatment effects, we also believe that Accutase increased viability rates due to quicker dissociation times. Accutase is a solution of proteolytic, collagenolytic enzymes without trypsin, was used for enzymatic dissociation of neural stem cell cultures (Wachs et al., 2003). Using Accutase for passaging cells reduced cell death and improved culturing conditions (Ruchi et al., 2008). The cooperation of ROCK inhibitor Y-27632 and Accutase dissociation significantly increase the survival rate of single hES cells in cryopreserved or regularly passaged cells.

We noted that undifferentiated and differentiated hES cells expressed similar mRNA amounts of the germ layer-specific genes neurofilament-68 (ectoderm) and enolase (mesoderm), however, this has also been reported in a previous study (Yoo et al., 2006). Moreover, we can find a small quantity of differentiated cells in Fig. 4B, as evident by their lighter immunostaining and irregular cell nuclei. Quantitative analysis of the hES cell surface markers (SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, OCT-4) by fluorescence-activated cell sorting also demonstrated that apparent undifferentiated hES cell colonies always contain small amounts of differentiated cells (Katkov et al., 2006; Liu et al., 2006; Ludwig et al., 2006; Sidhu and Tuch, 2006). The cryopreservation, passaging and maintenance culture of hES cells is always accompanied by a degree of spontaneous differentiation (Reubinoff et al., 2001; Fujioka et al., 2004; Richards et al., 2004; Zhou et al., 2004; Heng et al., 2006; Katkov et al., 2006; Li et al., 2008), which results from numerous accumulated subtle negative impacts induced by all manipulations in vitro. However, Y-27632 treatment does not induce the spontaneous differentiation of hES cells based on our present and previous data (Li et al., 2008), as well as other groups (Watanabe et al., 2007; Martin-Ibanez et al., 2008). We have not observed obvious adverse effects of Y-27632 treatment on pluripotent marker expression in maintenance culture even after a substantial number of passages. Although the H9 cell line was described in this study, Y-27632 treatment also had a similar effect on the cryopreservation and passaging of H1, CA1 and CA2 hES cell lines (data not shown). Further study is required to reveal whether subtle genetic alterations can be induced by the single cell cryopreservation, Accutase dissociation and Y-27632 treatment. In the present study, single hES cells can be effectively cryopreserved and propagated by incorporating the ROCK inhibitor Y-27632 and Accutase into the standard slow freezing and rapid thawing protocol in serum-free and feeder-free culture system. A recent study has also demonstrated that Y-27632 increased the survival rate of cryopreserved hES cells (Martin-Ibanez et al., 2008); however, we have demonstrated that Y-27632 treatment is also effective in serum/feeder-free conditions, and we have also described a partial mechanism for the increased survival observed in Y-27632-treated cultures. Furthermore, we have demonstrated that following Y-27632 treatment, the hES cells can be cryopreserved and passaged using similar methods to mouse ES cells and transformed cell lines. Throughout repeated freeze-thaw events and long-term culture, hES cells retain the key properties such as, typical morphological characteristics, marker expression (AKP, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, OCT-4), normal karyotype and the potential to differentiate into derivatives of all three germ layers. The convenient, reliable and effective cryopreservation and propagation of single hES cells provide a standard in vitro manipulation protocol and bring a bright future for the clinical application of hES cells.

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