ORIGINAL ARTICLE

Protein kinase A inhibitor, H89, significantly enhances survival rate of dissociated human embryonic stem cells following cryopreservation

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Abstract

Objectives: Human embryonic stem cells (hESCs) have huge potential for establishment of disease models and for treating degenerative diseases. However, the extremely low survival level of dissociated hESCs following cryopreservation is been a tremendous problem to allow for their rapid expansion, genetic manipulation and future medical applications. In this study, we have aimed to develop an efficient strategy to improve survival of dissociated hESCs after cryopreservation.

Materials and methods: Human embryonic stem cells (H9 line), dissociated into single cells, were cryopreserved using the slow-freezing method. Viable cells and their colony numbers in culture after cryopreservation were evaluated when treated with protein kinase A inhibitor H89. Western blotting was carried out to investigate mechanisms of low survival levels of dissociated hESCs following cryopreservation. Immunofluorescence, reverse transcription-polymerase chain reaction (RT-PCR), in vitro and in vivo differentiation were performed to testify to pluripotency and differentiation ability of hte cryopreserved cells treated with H89.

Results: H89 significantly improved survival level of dissociated hESCs after cryopreservation through ROCK inhibition. H89-treated cells still maintained their pluripotency and differentiation capacity.

Conclusions: This new approach for cryopreservation of single hESCs, using H89, can promote potential use of hESCs in regenerative medicine in the future.

1 | INTRODUCTION

Due to the unique ability to continuously self-renew and differentiate into ectodermal, mesodermal and endodermal lineages, human embryonic stem cells (hESCs) have become an excellent cell source for studying human development, establishing disease model and treating degenerative diseases.^{1–4} However, dissociated hESCs are susceptible to freezing damages and hardly recover after conventional cryopreservation,^{5–7} which is one of main challenges for their rapid expansion, genetic manipulation and future medical applications.^{8,9} Hence, it is

urgent to develop an efficient cryopreservation method with high survival rate of hESCs to meet their widespread applications in research and clinic.

Human embryonic stem cells can be cryopreserved for long-term storage using slow freezing and vitrification.¹⁰ Due to easy and scalable manipulations, slow freezing is widely used for dissociated hESC cryopreservation. However, the dissociated hESCs are extremely sensitive to stresses caused by dissociation and freezing during cryopreservation,^{11–13} such as mechanical, cold and osmotic stresses, leading to cell death during the following post-thaw culture after cryopreservation.^{14–17} Although various manipulations have been made to improve hESC survival, including utilizing new cryoprotective agents (CPAs) or different CPA combinations,^{18–20} controlled cooling

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rates^{21–23} and adherent status,^{24,25} a reliable method for cryopreserving hESCs with satisfactory survival rate is still required to be developed.

Dissociated hESCs during following culture after cryopreservation experience apoptosis, which has been thought to be the major reason for cell death and low survival rate.²⁶ Previous researches have made great efforts to elevate hESC survival.^{5,12,27,28} It has been demonstrated that the apoptosis inhibitors, including caspase inhibitor, Z-VAD-VMK and p53 inhibitor, and pifithrin- μ , are able to improve the survival rate of hESCs and human induced pluripotent stem cells (hiPSCs) after cryopreservation.^{6,29} A Rho-associated coiled-coil containing protein kinase (ROCK) inhibitor, Y-27632, and a vasodilator drug, pinacidil, have also been reported to significantly increase the survival rate of dissociated hESCs after cryopreservation.^{5,30–32} However, Y27632 is associated with aneuploidy^{33,34} and cell morphology changes.²⁷

In this study, the effects of H89, a selective and potent inhibitor of protein kinase A (PKA), in the different steps of cryopreservation, including before dissociation, during freezing and post-thaw culture, on the survival rate of single hESCs after cryopreservation were assessed. The cell morphology, undifferentiated status and differentiation capacity of dissociated hESCs during the following post-thaw culture were further evaluated.

2 | MATERIALS AND METHODS

2.1 | hESC maintenance culture

The hESCs (line H9) from WiCell Research Institute were used in this study. For feeder-free culture, hESCs were cultured on matrigel-coated plates using Essential 8 (E8) medium (Life Technologies, Carlsbad, CA, USA). For passaging, cells were digested using TrypLE™ Express (Life Technologies) and seeded at an appropriate density into matrigel-coated plates. For feeder-dependent culture, hESCs were seeded on the prepared plates coated with 0.1% gelatin (Sigma, St. Louis, MO, USA) and mitomycin C (10 μ g/ml) inactivated mouse embryonic fibroblast (MEF) cells in Dulbecco's modified Eagle's medium (DMEM)/F12 (Hyclone, Logan, UT, USA) supplemented with 20% KnockOut™-Serum Replacement (KSR; Gibco, Carlsbad, CA, USA), 1% MEM non-essential amino acids solution (Hyclone), 2 mM L-glutamine (Sigma, USA), 0.055 mM 2-mercaptoethanol (Gibco) and 10 ng/mL basic fibroblast growth factor (bFGF) (Life Technologies). The culture medium was changed every day. For passaging, hESCs were digested by collagenase IV (Gibco) and transferred into MEF-coated plates.

2.2 | H89 pre-treatment before hESC dissociation

For H89 pre-treatment before dissociation, hESCs were incubated with culture medium containing 3 μ M H89 (Selleck, Houston, TX, USA) for 2 hours. The hESCs without H89 treatment were used as the comparison. hESCs pre-treated with H89 before dissociation were marked as P(+) group, otherwise as P(-).

2.3 | Cryopreservation of hESCs

The dissociated hESCs by TrypLE express were re-suspended in E8 culture medium, equally mixed with the freezing solution (2x) containing 20% Me₂SO in E8 medium at 4°C for 20–30 minutes, kept at -80°C in a freezing container (Nalgene, Rochester, NY, USA) overnight and finally transferred to liquid nitrogen. For the cryopreservation of hESCs in the presence of H89, 6 μ M of H89 was added to the 2x freezing solution. The groups cryopreserved with or without H89 were marked as F(+) and F(-), respectively.

2.4 | Post-thaw culture of hESCs

After 3–7 days of cryopreservation, hESCs were thawed at 37°C in a water bath and then plated on the matrigel-coated 96-well plates for feeder-free culture or inactivated MEF-coated 96-well plates for feeder-dependent culture at different seeding densities ranging 0.1–4 $\times 10^4$ cells/well. Different concentrations of H89 from 0 to 10 μ M were added to the culture medium at day 1 or the whole course of post-thaw culture.

2.5 | AlamarBlue assay

After 1–4 days of culture, the alamarBlue assay was used to estimate the viable cells for the feeder-free culture. The alamarBlue reaction solution (Life Technologies) was added to the culture medium at the ratio of 1:10 and then incubated with cells at 37°C for 3 hours before detection using a microplate reader (Tecan, Switzerland).

2.6 | Alkaline phosphatase (AP) staining

Colony formation of hESCs was visualized using AP staining. The cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde (Sigma, Dorset, UK) for around 30 minutes and finally stained with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP-NBT) (Solarbio, Beijing, China) at room temperature for at least 2 hours in the dark. Images were taken using a digital camera.

2.7 | Western blot

The cryopreserved single hESCs were thawed and seeded into the matrigel-coated 35 mm dishes in E8 medium supplemented with 3 μ M H89 or 10 μ M Y27632. The cells were rinsed by PBS and then lysed for protein extraction at the indicated time points. Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking with 5% (w/v) skimmed milk, the membranes were incubated with PKA catalytic subunit $\alpha/\beta/\gamma$ (PKA $\alpha/\beta/\gamma$, Santa Cruz, USA), phosphorylated PKA $\alpha/\beta/\gamma$ (p-PKA $\alpha/\beta/\gamma$, Thr198), MYPT1, p-MYPT1 (Thr696), MLC2 and p-MLC2 (Thr18/Ser19) polyclonal antibodies (Ruiyingbio, Suzhou, China) at 4°C overnight, washed with TBST and followed by the incubation with HRP-conjugated IgG at

the dilution of 1:20 000 for 1 hour at room temperature. Bands were visualized using chemiluminescent detection (ECL; Thermofisher, Waltham, MA, USA). β -actin was used as the loading control. The Western blot analysis was performed at least three times.

2.8 | In vitro embryoid body (EB) formation and cardiomyocyte differentiation

To generate EBs, hESC colonies were dissociated using collagenase IV, suspended in hESC feeder-dependent medium without bFGF and then transferred into 35 mm ultra-low attachment Petri dishes for 4–7 days of suspension culture. The culture medium was changed every other day. For cardiomyocyte differentiation, the EBs were placed onto 0.1% gelatin-coated dishes to induce spontaneous differentiation for at least 7 days.

2.9 | In vitro neural progenitor cells (NPCs) induction

The H89-treated hESCs were dissociated by TrypLE Express, plated on matrigel-coated 24-well plates at the density of 2.5×10^5 cells/cm² and cultured with STEMdiff™ neural induction medium (STEMCELL, Vancouver, Canada). After 6 days of culture, the cells were passaged at a density of $1.25\text{--}2 \times 10^4$ cells/cm² until passage 3.

2.10 | Immunocytochemical characterization

Cells grown on glass coverslips were fixed with 4% paraformaldehyde at room temperature for 20 minutes, permeabilized with 1% (v/v) Triton X-100 (Sigma, USA) supplemented with 1% (w/v) bovine serum albumin (BSA) (Sigma, USA) in PBS at room temperature for 30 minutes, and blocked with 1% BSA in PBS at 37°C for 60 minutes. Primary antibodies, goat anti-OCT4 (octamer-binding transcription factor 4) polyclonal antibody (Santa Cruz, USA), mouse anti-SSEA4 (stage-specific embryonic antigen 4) monoclonal antibody (R&D, Minneapolis, USA), goat anti-NANOG polyclonal antibody (R&D), mouse anti-SOX2 (sex determining region Y-box 2) monoclonal antibody (Chemicon, Temecula, CA, USA) and rabbit anti- α -actinin/Troponin T2 (TNNT2)/ β -III Tubulin/ Paired box 6 (PAX6) (ABclonal, Cambridge, MA, USA) polyclonal antibodies were added to the cells at the recommended concentrations in PBS at 4°C overnight followed by PBS rinsing to remove the unbound primary antibodies. The secondary antibodies, Alexa Fluor (AF) 594 conjugated donkey anti-mouse or anti-goat IgG with the dilution of 1:500 and AF488 conjugated donkey anti-mouse for SSEA4 and anti-rabbit (Molecular Probes, Carlsbad, CA, USA) for α -actinin/TNNT2/ β -III Tubulin/PAX6 diluted at 1:200 in PBS were applied to the cells at 37°C for 60 minutes in the dark. After washing with PBS for three times, the cells were visualized using a fluorescence microscopy (Nikon, Tokyo, Japan) connected to a CCD (Tucsen, Fuzhou, China).

2.11 | Karyotype analysis

Karyotype analysis of the cryopreserved hESCs treated with H89 for at least 10 passages was carried out using the G-banding method as

described previously with slight modification.¹² Briefly, cells were incubated with 0.06 μ g/mL of colcemid at 37°C for 4 hours, trypsinized, re-suspended and then incubated in 0.56% KCl at 37°C for 20 minutes. The collected cells by centrifugation were fixed with methanol:glacial acetic acid (3:1) for three times and then dropped onto pre-cooled slides to spread the chromosomes. The dried slides were treated with 0.025% trypsin for 90 seconds and stained with Giemsa.

2.12 | Reverse transcription-polymerase chain reaction (RT-PCR)

Normal hESCs, EBs, the differentiated cardiomyocytes and NPCs were prepared, and the total RNA extraction and cDNA synthesis were performed as manufacturer's instructions using Trizol reagent (TaKaRa, Dalian, China) and cDNA first-strand synthesis mix (Transgen, Beijing, China), respectively. The primers used for RT-PCR were listed as the supporting information (Table S1).

2.13 | Teratoma formation assay

The hESCs (treated with H89 more than 10 passages) were dissociated by collagenase IV and injected subcutaneously into Beige-SCID mice. Eight weeks later, the resulting tumours were removed, fixed with 4% formaldehyde in PBS and embedded in paraffin. Sections were stained with haematoxylin and eosin (HE).

2.14 | Statistical analysis

At least three independent experiments were carried out for each condition. Data were presented as mean $\pm$ SD. The statistical significance was assessed using the Student's one-way ANOVA. A probability of $P < .05$ was considered to be significant.

3 | RESULTS

3.1 | Effects of H89 concentration and seeding density

To find out the effect of H89 on the survival of dissociated hESC after cryopreservation, various concentrations of H89 at 0, 1, 3, 6 and 10 μ M were introduced to the post-thaw culture medium at day 1. The cell growth was monitored using alamarBlue assay and AP staining for feeder-free and feeder-dependent culture, respectively. The reverse U shapes indicated that the optimal H89 concentration for improving hESC survival was 3 or 6 μ M for both feeder-dependent and feeder-free culture manners (Fig. 1a,d).

To determine the effect of cell seeding density on the hESC survival after cryopreservation, the thawed cells were initially seeded at the density of 10 000, 20 000 and 40 000 cells/well in 96-well plates for the feeder-free culture and 1000, 3000, 6000, 10 000 and 20 000 cells/well for the feeder-dependent culture. As shown in Fig. 1b,c and e, when 3 μ M H89 was applied on the first day of post-thaw culture, the viable cells was increased with the increasing cell seeding density for both the feeder-free and the feeder-dependent

FIGURE 1 The effects of H89 concentration and seeding density on the survival of the hESCs after cryopreservation. (a) The survival of hESCs at different H89 concentrations under feeder-dependent and feeder-free culture conditions. After 3 days for feeder-free culture and 4 days for feeder-dependent culture, the alamarBlue analysis and colony number counting (positive AP staining) were performed, respectively (*, $P < .05$). (b) The survival of hESCs at different seeding densities with 3 μM H89 in culture medium at day 1 (*, $P < .05$). The morphology of hESCs 2 days after post-thaw culture at different seeding densities and 1 day after post-thaw culture at different H89 concentrations under feeder-free culture condition were shown in (c) and (d), respectively. (e) The images of AP-positive colonies 4 days after feeder-dependent culture at different seeding densities and H89 concentrations

culture. Hence, the cell seeding density of 40 000 cells/well (96-well plates) for the feeder-free culture and 10 000 cells/well for the feeder-dependent culture was chosen for the further experiments.

3.2 | Effects of H89 treatment during different steps of cryopreservation

To determine the effects of H89 treatment during different steps of cryopreservation on hESC survival, a series of manipulations were performed, including with or without pre-treatment with 3 μM H89 before dissociation, $P(+/-)$, with or without H89 in the freezing solution, $F(+/-)$, and with or without the presence of H89 at day 1 of post-thaw culture, $H(+/-)$. Figure 2 shows the viable cells of dissociated hESCs after cryopreservation under different conditions. For the feeder-free culture, the results revealed that both the incubation with H89 before

dissociation and H89 addition in the freezing solution significantly increased the number of viable cells and colonies (Fig. 2a,b, $P < .05$). The cell survival was further improved by the addition of H89 in the freezing solution when the cells were pre-treated with H89 before dissociation, although not significant. Interestingly, the addition of H89 to the post-thaw culture medium at day 1 dramatically increased the viable cells and colonies for all the tested conditions without significant difference between different tested conditions (Fig. 2a,b).

Compared with the feeder-free culture, the H89 pre-treatment before dissociation and the addition of H89 in the freezing solution had no significant effect on the cell survival, showing the parallel colony number with the control group (Fig. 2c,d). In contrast, the cell colony number was significantly increased by around three-folds for all the tested conditions due to the introduction of H89 in the post-thaw culture medium at day 1.

FIGURE 2 The effects of H89 addition before dissociation, during freezing and post-thaw culture of the cryopreserved hESCs on the cell survival. P(+)/(-) represents H89 pre-treatment or not before the dissociation of hESCs. F(+)/(-) notes the presence or absence of H89 in the cryoprotective medium. H(+)/(-) indicates the addition of H89 or not in the post-thaw culture medium. (a) The survival of hESCs cryopreserved under different conditions. The hESC survival was determined by alamarBlue assay after 3 days of post-thaw culture under feeder-free condition. (b) The morphology of hESCs cryopreserved under different conditions 1 day after plating. (c) The survival of hESCs cryopreserved under different conditions with feeder-dependent culture. The AP-positive colonies were counted after 5 days of post-thaw culture and the images of AP-positive colonies were shown in (d). The mark of “*” means the significant difference in the cell survival between the groups with and without H89 treatments during the post-thaw culture

3.3 | Effect of continuous H89 treatment during post-thaw culture of hESCs

We further looked into the effects of the continuous H89 treatment during the whole course of post-thaw culture on the cell survival of dissociated hESCs after cryopreservation for both feeder-dependent and feeder-free culture. The result showed that continuous H89 treatment during post-thaw culture did not increase the viable cells and colonies further for the feeder-free culture (Fig. 3a,b), even led to slightly fewer viable cells and colonies compared with 1 day post-thaw culture with H89 for the feeder-dependent culture (Fig. 3c,d).

3.4 | H89 enhances the survival of hESCs after cryopreservation through ROCK inhibition

To investigate the mechanism behind the enhancement of cell survival contributed by H89, Western blot analysis was performed. The experimental results revealed that the phosphorylation of PKA (p-PKA) in hESCs after thawing was significantly and continuously increased from 10 minutes to 2 hours (Fig. 4a). Similarly, the phosphorylation of MLC2 (p-MLC2) and MYPT1 (p-MYPT1), two substrates of ROCK, was markedly elevated, showing a steady increase from 10 minutes

to 2 hours and the highest at 30 minutes after thawing, respectively. When the thawed hESCs were incubated with H89 for 30 minutes, H89 obviously diminished the cryopreservation-induced phosphorylation of PKA (Fig. 4b). Importantly, H89 also attenuated the levels of p-MLC2 and p-MYPT1, even stronger than Y27632.

3.5 | Characterization of H89-treated hESCs

To determine whether H89 treatment affects the pluripotency and differentiation capacity of hESCs, we cultured the thawed hESCs with H89 at day 1 of each passage for more than 10 passages. As shown in Fig. 5a, four key pluripotent markers of hESCs, including the nuclear markers OCT4, SOX2 and NANOG, and the surface marker SSEA4, exhibited highly positive expression, showing that H89-treated hESCs still maintained their undifferentiated status. In addition, H89 treatment did not affect the genetic stability of hESCs (Fig. 5b).

To evaluate the differentiation potential of H89-treated hESCs, the hESCs after cryopreservation with H89 treatment for more than 10 passages were used for *in vitro* differentiation. As shown in Fig. 5c, the H89-treated hESCs successfully formed EBs *in vitro*, which further derived into three germ layers (ectoderm, mesoderm and endoderm). Furthermore, the H89-treated hESCs were directly induced to differentiate into

FIGURE 3 The effects of continuous treatment of H89 on the hESC growth during the post-thaw culture. The survival of hESCs under feeder-free and feeder-dependent culture conditions determined by alamarBlue assay and AP-positive colony counting is shown in (a) and (c), respectively. The morphology of hESCs post-thaw cultured for 2 days under feeder-free condition and for 3 days under feeder-dependent condition is shown in (b) and (d), respectively (*, $P < .05$)

NPCs (Fig. 5c). RT-PCR results confirmed the expression of three germ layer genes in hESCs, EBs and NPCs (Fig. 5d). The ectoderm markers, sex determining region Y (SRY)-box 1 (SOX1) and paired box 6 (PAX6) exhibited the highest expression in 7-day EBs and in NPCs for PAX6, respectively. The mesoderm marker, peroxisome proliferator-activated receptor (PPAR), was highly increased in EBs and NPCs. The other mesoderm markers, cardiac troponin T2 (TNNT2) and Brachyury (T), and the endoderm markers, albumin (ALB), α -fetoprotein (AFP), sex determining region Y (SRY)-box 17 (SOX17) and forkhead box A2 (FOXA2), showed the highest levels in 16-day EBs. The resulting EBs were further able to spontaneously differentiate into cardiomyocytes according to the immunofluorescence staining of the cardiomyocyte markers, TNNT2 and α -actinin (Fig. 5e). In addition, the H89-treated hESCs efficiently differentiated into NPCs with the high expression of the neuron marker, β -III tubulin (Tuj1), and the NPC marker, PAX6 (Fig. 5f). For in vivo differentiation, the teratomas were obtained, showing the cells of three germ layers visualized by the HE staining, including epithelium in endoderm, cartilage in mesoderm and neural tissue in ectoderm (Fig. 5g).

4 | DISCUSSION

In this study, the effects of H89 treatment before dissociation, during freezing and following post-thaw culture on enhancing the survival of dissociated hESC during following culture after cryopreservation were investigated. A new cryopreservation protocol of dissociated hESCs to increase the cell survival using slow freezing with H89 treatment was developed, suitable for both feeder-dependent and feeder-free culture. The developed cryopreservation approach may promote the potential use of hESCs in regenerative medicine in the future.

Cell seeding density is an important factor to maintain cell survival and proliferation during culture.^{35,36} At a low seeding density, it may be difficult for cells to interact with each other, especially for hESCs which require close contacts between cells.³⁷ By contrast, a high seeding density makes cells much easier to re-aggregate and get in touch with each other.³⁸ Here, hESCs at the low cell seeding density survived

FIGURE 4 The effects of H89 on PKA and ROCK inhibition analysed by Western blot. (a) Western blot analysis of the phosphorylation levels of PKA, MLC2 and MYPT1, as well as their total levels in hESCs after thawing. (b) Western blot analysis of the phosphorylation levels of PKA, MLC2 and MYPT1, as well as their total levels in hESCs after thawing when treated with H89. The samples immediately after thawing (0 h) were used as the negative controls (without any inhibitor treatment) and the groups treated with 10 μ M Y27632 as the positive controls. β -actin was used as the loading control

FIGURE 5 The pluripotency and differentiation ability of hESCs after cryopreservation and post-thaw culture with H89 treatments for at least 10 passages. (a) The immunofluorescence for the pluripotent markers of hESCs. DAPI was used for nucleus staining. (b) The karyotype analysis of the hESCs treated by H89. (c) The morphology of hESCs and the in vitro differentiation of hESCs into EBs, ectoderm, mesoderm, endoderm and NPCs. (d) RT-PCR analysis of the key markers of three germ layers in the undifferentiated and differentiated cells. β -actin served as an internal control. Immunofluorescence analysis of the cardiomyocytes and NPCs derived from hESCs is shown in (e) and (f), respectively. (g) The teratoma formation of hESCs. The teratomas showed the cells of three germ layers visualized by the HE staining, including epithelium in endoderm, cartilage in mesoderm and neural tissue in ectoderm.

and proliferated for the feeder-dependent culture, but the high cell seeding density was required for the feeder-free culture (Fig. 1), probably due to the differences in matrixes and growth factors in the living circumstances³⁹ leading to the differences in cell responses.

We further determined the effects of H89 treatment applied in different steps of cryopreservation on hESC survival. The H89 pre-treatment before dissociation and H89 in the freezing solution for the feeder-free culture have positive effects on the cell survival

(Fig. 2a), implicating that the early protection of hESCs from damages during dissociation may be helpful for decreasing the hESC death during cryopreservation. However, the introduction of H89 in all the procedures of cryopreservation did not lead to more cell survival compared with the group with H89 only in the culture medium. As demonstrated in the previous studies, the cell death of dissociated hESCs during the following post-thaw culture after cryopreservation is caused by apoptosis but not necrosis.^{9,26,40} Our result draws a consistent conclusion since around 90% of the hESCs remain alive immediately after thawing (Fig. S1). The massive cell death mainly occurs during the following culture after thawing. Therefore, the negligible superposed effects of simultaneous treatment with H89 during each stage of the process (Fig. 2) implicate that the apoptosis signals may gradually develop in cells during the each step of cryopreservation and reach the maximum level until the post-thaw culture. The experimental results indicate that the early protection resulting from H89 present in both before dissociation and during freezing is able to enhance the hESC survival after cryopreservation, while adding H89 in the stage of post-thaw culture alone may inhibit all the apoptosis events caused by dissociation and freezing.

For the feeder-dependent culture, it is interesting to notice that the application of H89 before dissociation and during freezing did not promote the colony formation, while the introduction of H89 at day 1 of post-thaw culture enhanced the colony formation (Fig. 2c). This result further suggested that hESCs are very sensitive to culture microenvironments,⁴¹ which are not only affected by various growth factors⁴² but also by physical structures.^{43,44} The differences in microenvironments may further result in differential gene expression, migration and cell-cell contacts,^{37,45,46} which partly explain the different performances when hESCs were cultured with feeder and without feeder. Even so, the simple addition of H89 in the post-thaw culture medium can achieve the high survival rate of dissociated hESCs after cryopreservation for the feeder-free culture.

To examine the effects of the continuous H89 treatment on cell growth, the culture medium containing 3 μ M H89 was applied during the whole course of post-thaw culture. However, no better results were observed (Fig. 3), revealing that the introduction of H89 at day 1 of post-thaw culture is sufficient for enhancing the survival rate of cryopreserved hESCs. These results suggested that the apoptosis of dissociated hESCs during the following culture after cryopreservation may mainly occur at the early stage of post-thaw culture, consistent with the previous study.⁴⁰

H89 attenuated the level of p-PKA (Fig. 4b) which is involved in different mechanisms causing cell apoptosis or anti-apoptosis in various cell lines,^{47–52} implicating that PKA activation may play an important role in cryopreservation-induced cell death of hESCs. In addition, both of p-MLC2 and p-MYPT1 in hESCs after thawing were markedly elevated (Fig. 4a), a proof for the activation of ROCK signalling pathway. The application of H89 during the post-thaw culture led to the inhibition of p-MLC2 and p-MYPT1, similar with the effect of Y27632 (Fig. 4b) which can promote hESC survival

through ROCK inhibition by suppressing p-MLC2 and p-MYPT1.⁴⁰ These results indicated that H89 depresses PKA activation and promotes hESC survival after cryopreservation through ROCK inhibition. H89 also elevates dissociated hESC survival through ROCK inhibition as reported in our previous study.⁵³ Therefore, we deduce that the increased survival of hESCs by H89 addition in the different stages of cryopreservation, including before dissociation, during freezing and following thawing (Fig. 2), may also benefit from the similar mechanism. The role of H89 and PKA in the different steps of cryopreservation as well as their mechanisms should be further determined.

In conclusion, a simple and effective approach was developed to enhance the survival of dissociated hESCs after cryopreservation without affecting their pluripotency and in vitro differentiation ability. The hESCs after H89 treatment still have stable karyotype during the long-term culture. Therefore, this new approach for the cryopreservation of single hESCs with H89 may promote the potential use of hESCs in regenerative medicine in the future.

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ABBREVIATIONS

AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; EB, embryoid body; hESCs, human embryonic stem cells; NBT, nitro blue tetrazolium; NPCs, neural progenitor cells; PKA, protein kinase A.

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SUPPORTING INFORMATION

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