Stem Cell Rev and Rep (2010) 6:215–223
DOI 10.1007/s12015-010-9118-5

The ROCK Inhibitor Y-27632 Negatively Affects the Expansion/Survival of Both Fresh and Cryopreserved Cord Blood-Derived CD34+ Hematopoietic Progenitor Cells

Y-27632 negatively affects the expansion/survival of CD34 + HSPCs

Clara Bueno · Rosa Montes · Pablo Menendez

Published online: 24 February 2010

Abstract Cord blood (CB) is an unlimited source of hematopoietic stem and progenitor cells (HSPC). The use of cryopreserved CB-derived CD34+ HSPCs is successful in children and usually leads to rapid hematopoietic recovery upon transplantation. However, current methods for ex vivo expansion of HSPCs still result in a loss of multilineage differentiation potential and current freeze-thawing protocols result in significant cell death and loss of CD34+ HSPCs. The major cause for the loss of viability after slow freezing is apoptosis induced directly by cryoinjury. Very recent reports have demonstrated that Y-27632, a selective and robust ROCK inhibitor is a potent inhibitor of the apoptosis and is efficient in enhancing the post-thaw survival and recovery of different human stem cells including human embryos, hESCs, induced pluripotent stem cells and mesenchymal stem cells. Here, we analyzed the effect of such an inhibitor in CB-derived CD34+ HSPCs. CB-derived CD34+ HSPCs were MACS-isolated and treated with or without 10 μM of Y-27632. The effect of Y-27632 on culture homeostasis was determined in both fresh and cryopreserved CB-derived CD34+ HSPCs. Our results indicate that the Y-27632 not only dramatically inhibits cell expansion of both fresh and cryopreserved CD34+ HSPCs but also impairs survival/recovery of CD34+ HSPCs upon thawing regardless whether Y-27632 is added to both the cryopreservation and the expansion media and or just to the expansion culture medium with or without hematopoietic cytokines. This study identifies for the first time a detrimental effect of Y-27632 on the expansion and survival of both fresh and cryopreserved CB-derived CD34+ HSPCs, suggesting that Y-27632 may have a differential impact on distinct lineage/tissue-specific stem cells. Our data suggest different functions of Y-27632 on human stem cells growing in suspension versus those growing attached to either treated tissue culture plastic or extracellular matrix. We discourage any clinical application of Y-27632 in potential technical developments aimed at improving cryopreservation procedures of CB-derived cells and/or in vitro expansion of HSPCs without spontaneous differentiation.

Keywords Human CB-CD34+ HSPCs · Rock inhibitor Y-27632 · Cryopreservation · Survival · Expansion

Introduction

Hematopoietic stem cell transplantation (HSCT) may be curative in a large variety of diseases. Cord blood is an unlimited source of hematopoietic stem and progenitor cells (HSPC) for allogeneic HSCT. Cord blood transplantation (CBT) has extended the availability of allogeneic HSCT to patients who would not otherwise be eligible for this curative approach [1]. CB units are usually banked for future unrelated or related CBT. There are currently over 400.000 banked CB units registered worldwide [1]. The use of cryopreserved CB-derived CD34+ HSC is successful in

Clara Bueno and Rosa Montes contributed equally to this work.

C. Bueno · R. Montes · P. Menendez
Andalusian Stem Cell Bank, Centro de Investigación Biomédica, Consejería de Salud-Universidad de Granada, Granada, Spain

C. Bueno (✉) · P. Menendez (✉)
Andalusian Stem Cell Bank, Instituto de Investigación Biomédica, Avda del Conocimiento, Armilla, Granada 18100, Spain
e-mail: clara.bueno@juntadeandalucia.es
e-mail: pablo.menendez@juntadeandalucia.es

children and usually leads to rapid hematopoietic recovery [2–5]. However, there is clearly room for improvements as current methods for ex vivo expansion of HSPC still result in a loss of multilineage differentiation potential and current freeze-thawing protocols result in significant cell death and loss of CD34+ HSPC populations essential for engraftment [6–11].

The major cause for the loss of viability after slow freezing is apoptosis induced directly by cryoinjury [12]. Very recent reports have demonstrated that Y-27632, a selective and robust inhibitor of p160-Rho-associated coiled-coil kinase (ROCK), is a potent inhibitor of the apoptosis and is efficient in enhancing the post-thaw survival and recovery of frozen human embryos [13], cryopreserved human embryonic stem cells (hESCs) [14–20], induced pluripotent stem cells (iPS cells) [21] and human mesenchymal stem cells (hMSCs) [22].

In the present study, therefore, we attempted to assess the effect of the ROCK inhibitor Y-27632 on the expansion and cellular survival of both fresh and cryopreserved CD34+ HSPCs which, in contrast, to the aforementioned human stem cells (blastocysts, hESCs, iPS cells, MSCs) grow in suspension rather than attached or as colony aggregates.

Material and Methods

Cord Blood-Derived $CD34^+$ HSPC Isolation and Culture

Different fresh Cord Blood (CB) units from healthy newborns were obtained from local hospitals upon approval by our local Ethics and Biozahard Board Committee. CB samples were pooled to reduce variability between individual freshly-isolated CB units (n=5 CB pools). Mononuclear cells were isolated using Ficoll-Hypaque (GE Healthcare, Stockholm, Sweden). After lysing the red blood cells (Lysis solution, StemCell Technologies, Vancouver, Canada), $CD34^+$ cells were purified by magnetic bead separation using the human CD34 MicroBead kit (Miltenyi, Munich, Germany) and the AutoMACS Pro separator (Miltenyi) as per manufacturer's instructions. After washing in phosphate-buffered saline (PBS), equal numbers of $CD34^+$ cells were plated in liquid culture: Stem Spam (Stem Cell Technologies) supplemented with SCF (100 ng/mL), FLT3L (100 ng/mL) and IL-3 (10 ng/mL) (Peprotech, London, UK) in the presence or absence of 10 μM of the ROCK inhibitor Y-27632 (Calbiochem, San Diego, CA, USA). In some experiments, the liquid culture was not supplemented with hematopoietic cytokines to address the potential effect of the ROCK inhibitor in the absence of such cytokine stimulation. Y-27632 was added to the culture medium daily.

As aforementioned, identical experiments (n=4) were performed using MACS-isolated CB-derived $CD34^+$ cells from cryopreserved CB units. CB-derived cells were frozen using current protocols (culture medium supplemented with 10% *v/v* dimethyl sulfoxide (DMSO) using a slow linear cooling curve). Ten μM of Y-27632 was added to both the cryopreservation medium and the expansion medium or just to the expansion medium daily. Cells were kept frozen in liquid nitrogen for several weeks. Then, cells were thawed at 37°C, washed in PBS and equal numbers of $CD34^+$ cells were cultured for up to 13 days in the presence or absence of 10 μM of Y-27632. Analysis of CD34+ cells in the culture was determined by flow cytometry at days 0, 4, 9 and 13.

Cell Cycle Analysis

CB-derived $CD34^+$ cells were harvested after 4, 9 and 13 days of culture and pelleted by centrifugation. Cell pellets were washed with PBS, fixed in 70% ice-cold ethanol, and stored for up to 2 weeks at −20°C. Cells were washed with PBS followed by incubation in 50 μg/ml propidium iodide (PI) and 100 μg/ml RNase A (Sigma) for 30 min. Stained nuclei were analyzed on a FACS Canto-II using the FACS Diva software (Becton Dickinson, San Jose, CA, USA). Modfit software (Becton Dickinson) was used to discriminate among apoptotic cells (Sub-G_0/G_1), quiescent cells (G_0/G_1) and cycling cells ($S/G_2/M$). Cell death was also analysed by 7-actinomycin D (7-AAD) staining [23].

Caspase 3 and PI Staining

CB-derived $CD34^+$ cultured cells were harvested after 4, 9 and 13 days and pelleted by centrifugation. Unfixed cells were assayed for active caspase 3 immediately after harvesting using CaspGLOW Fluorescein Active Caspase-3 staining Kit according to the manufacturer´s instructions (MBL Corp., Woburn, MA). Subsequently, they were resuspended in PBS containing 50 μg/ml of PI, 100 μg/ml of RNAse A and 0.1% (*v/v*) of Triton X-100. After 30 min incubation, the cell suspension was analyzed by flow cytometry for active caspase-3 and cell cycle simultaneously on a FACS Canto-II (Becton Dickinson).

Colony Forming Unit (CFU) Assay

On day 0, 4, 9, 13 of culture, CB-derived $CD34^+$-enriched fraction was plated ($2\times10^3/cm^2$) in methycellulose assays supplemented with SCF (50 ng/mL), GM-CSF (10 ng/mL), IL-3 (10 ng/mL) and Erytropoietin (3U/mL) (Methocult GF H4434; StemCell Technologies) in the presence or absence of Y-27632 (10 μM). After 12–14 days in culture, colonies were counted and scored [3, 24–26].

Statistical Analysis

All data are expressed as mean ± standard errors of the mean (SEM). Statistical comparisons were performed with a paired Student's *t* test. Statistical significance was defined as a *P* value <0.05 [4, 5, 27, 28].

Results

Y-27632 Dramatically Inhibits Proliferation But Do Not Impair Differentiation of Freshly-Isolated CD34+ HSPCs

CD34+ HPSCs were MACS-isolated from fresh CB units. Purity was consistently higher than 91% (Fig. 1a). Equal numbers of CD34+ HSPCs were cultured in liquid culture supplemented with hematopoietic cytokines for up to 13 days in the presence or absence of Y-27632. Proliferation was measured on days 4, 9 and 13 relative to the number of CD34+ HSPCs initially plated. As shown in Fig. 1b, overall cell expansion was reduced up to 8-fold ($p<0.001$) when Y-27632 was added into the culture medium. As it is well-established, CD34+ HSPCs gradually differentiated in vitro losing CD34 expression, indicating that Y-27632 does not impair differentiation/maturation of CD34+ HSPCs (Fig. 1c, d). Accordingly, the absolute numbers of both CD34+ and CD34- cell subsets throughout the 13-day period in vitro were measured in the presence or absence of Y-27632. As shown in Fig. 1e and f, Y-27632 significantly ($p<0.03$) inhibited the proliferation of CD34+ HSPCs and, at least extend, CD34- mature cells (Fig. 1e, f). Interestingly, Y-27632 inhibited proliferation of CD34+ HPSCs (Fig. 1e) but did not impair the differentiation/maturation of CD34+ HSPCs in liquid culture (Fig. 1c, d) or in colony-forming units (CFU) assays (Fig. 1g). Taken together, this data indicates that Y-27632 dramatically inhibits the proliferation of freshly-isolated CD34+ HSPCs without impairing CD34+ HSPCs differentiation/maturation.

Y-27632 Induces Cell Death Coupled to Cell Cycling of CD34+ HSPCs

Because of the robust loss of cellularity in CB cell cultures treated with Y-27632, we next analysed cell death and cell cycle distribution in Y-27632-treated and untreated CB cultures. Cell death was determined as the Sub G_0/G_1 fraction (Fig. 2a) or 7-ADD+ fraction. Both assays provided identical results (data not shown). As shown in Fig. 2b, cell death was significantly higher (between 2–3 fold higher) in those cultures treated with Y-27631: 14% vs 5%, 41% vs 18% and 37% vs 18% on day 4, 9 and 13, respectively. As for cell cycle distribution, no differences were observed at day 4 after Y-27632 treatment (Fig. 2c, d). From day 9 onwards, a significantly higher (1.45 fold higher) number of cycling cells ($S/G_2/M$) were detected in those CB-cultures treated with Y-27632: 44% vs 30%, and 32% vs 22% on day 9 and 13, respectively. CB-derived cells were stained with an antibody anti active Caspase 3 and PI in order to determine at which phase of the cell cycle apoptosis is mainly occurring. As depicted in Fig. 2e, over 80% of the caspase-3+ apoptotic cells were at G_0/G_1 cell cycle phase in Y-27632-treated cultures. These data suggest that Y-27632 dramatically inhibits CD34+ HSPCs expansion through apoptosis/cell death induction which contributes more than cell cycling (2–3 fold versus 1.45 fold) to Y-27632-induced inhibition of proliferation.

We next wondered whether the Y-27632 effect may be linked, to some extend, to the cytokine cocktail (SCF, FLT3, IL3) used to pre-stimulate CB-derived CD34+ HSPCs. Accordingly, the same cell cycle/apoptosis assay was performed under cytokine-free conditions (Fig. 2f). In hematopoietic cytokines-free cultures, Y-27632 similarly inhibited cell expansion by inducing cell death (Sub G_0/G_1: 71% vs 58%). Taken together, regardless the use of hematopoietic growth factors, Y-27632 robustly inhibits cell proliferation of freshly-isolated CD34+ HSPCs by inducing apoptosis/cell death.

Y-27632 Improves Neither Expansion Nor Survival of Cryopreserved CD34+ HSPCs

In an autologous transplantation setting, previously harvested and frozen CD34+ HSPCs need to be thawed before the infusion into the patient. The major cause for the loss of viability after slow freezing is apoptosis induced directly by cryoinjury [22]. Very recent reports have demonstrated that Y-27632, is efficient in enhancing the post-thaw survival and recovery of different cryopreserved human stem cells when added either to the cryopreservation medium or expansion medium or to both [13, 15–20]. We thus wanted to determine the effect of Y-27632 on cryopreserved CB-derived CD34+ HSPCs. Y-27632 was added to both the cryopreservation and the expansion media or just to the expansion medium.

An initial set of experiments was performed in which the effect of Y-27632 addition was analysed only in the expansion culture medium used to grow and maintain thawed CD34+ HSPCs. Identical with the effect of Y-27632 on freshly-isolated CD34+ HSPCs, overall cell expansion of frozen-thawed CB-derived cells was profoundly reduced (10-fold; $p<0.0001$) from day 4 onwards when Y-27632 was added into the medium (Fig. 3a). As reported for freshly-isolated CD34+ HSPCs, frozen-thawed CD34+ cells also differentiated normally in vitro, losing CD34 expression within 13 days (Fig. 3b, c). Accordingly, regarding absolute numbers, Y-27632 significantly inhibited the expansion of CD34+ HSPCs ($p<0.01$)

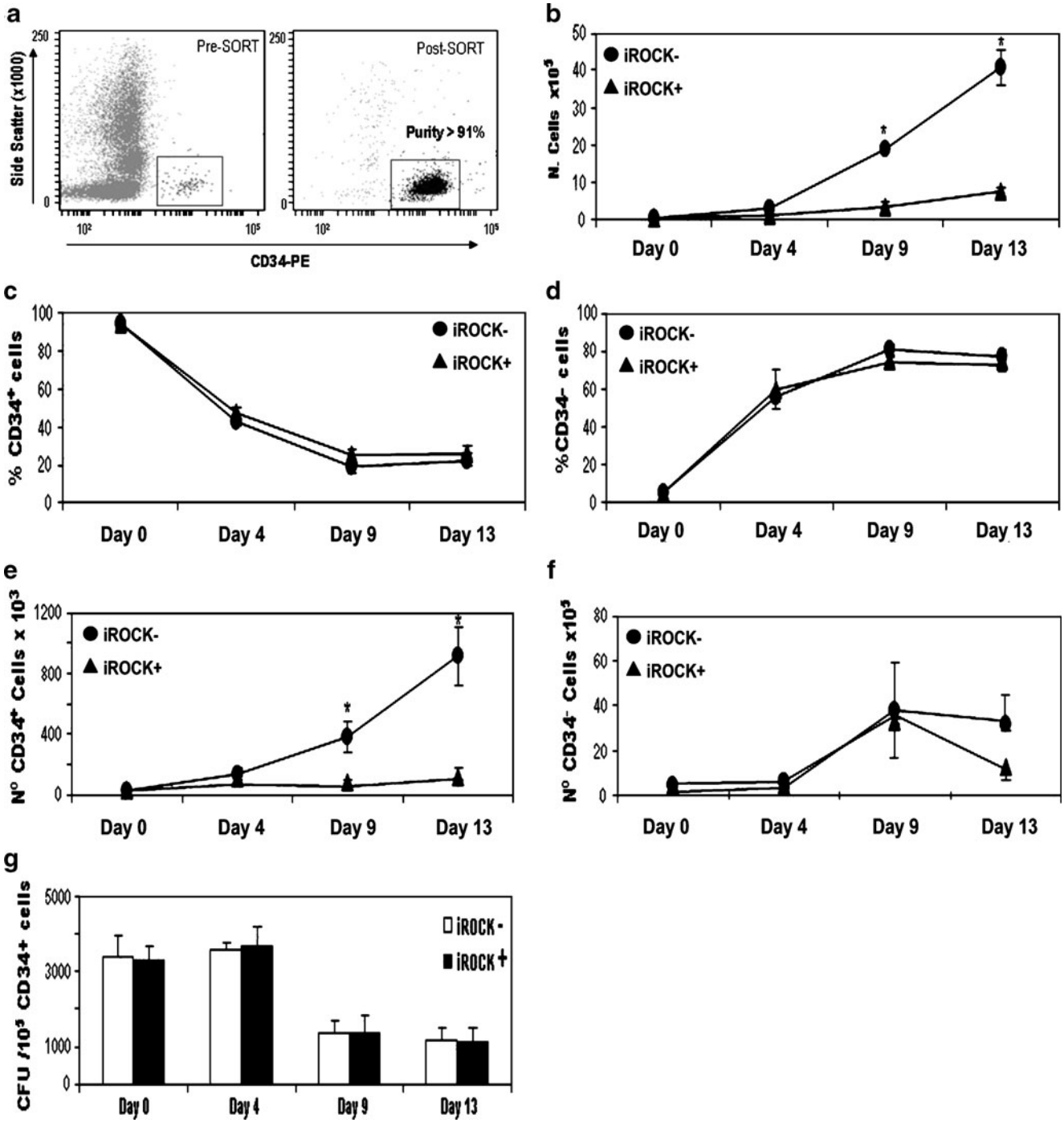

Fig. 1 Y-27632 inhibits cell expansion but not differentiation of freshly isolated CD34+ HSPCs. **a** Representative flow cytometry dot plots displaying purity upon CD34+ FACS sorting. **b** Growth curves (cell expansion) in the presence or absence of Y-27632 measured for up to 13 days. Percentage of CD34+ (**c**) and CD34- cells (**d**) in the culture at different time points in the presence or absence of Y-27632. Absolute numbers of CD34+ (**e**) and CD34- cells (**f**) at different time points in the presence or absence of Y-27632. **g** Total number of hematopoietic colonies per 10^5 CD34+ cells plated in methylcellulose assays in the presence or absence of Y-27632. Asterisks represent statistical significant differences ($p<0.05$)

(Fig. 3d) and CD34- mature cells ($p<0.001$) (Fig. 3e) throughout the 13-day period. Figure 3f shows a representative CD34 versus 7-ADD staining displaying the higher apoptotic rate (7-ADD+ cells) in both CD34+ and CD34- cell subsets.

We finally wondered whether the supplementation of the standard cryopreservation medium containing 10% *v/v* DMSO (slow linear cooling curve) with Y-27632 would facilitate the survival/recovery of cryopreserved CD34+ HSPCs upon thawing. Addition of Y-27632 to both the

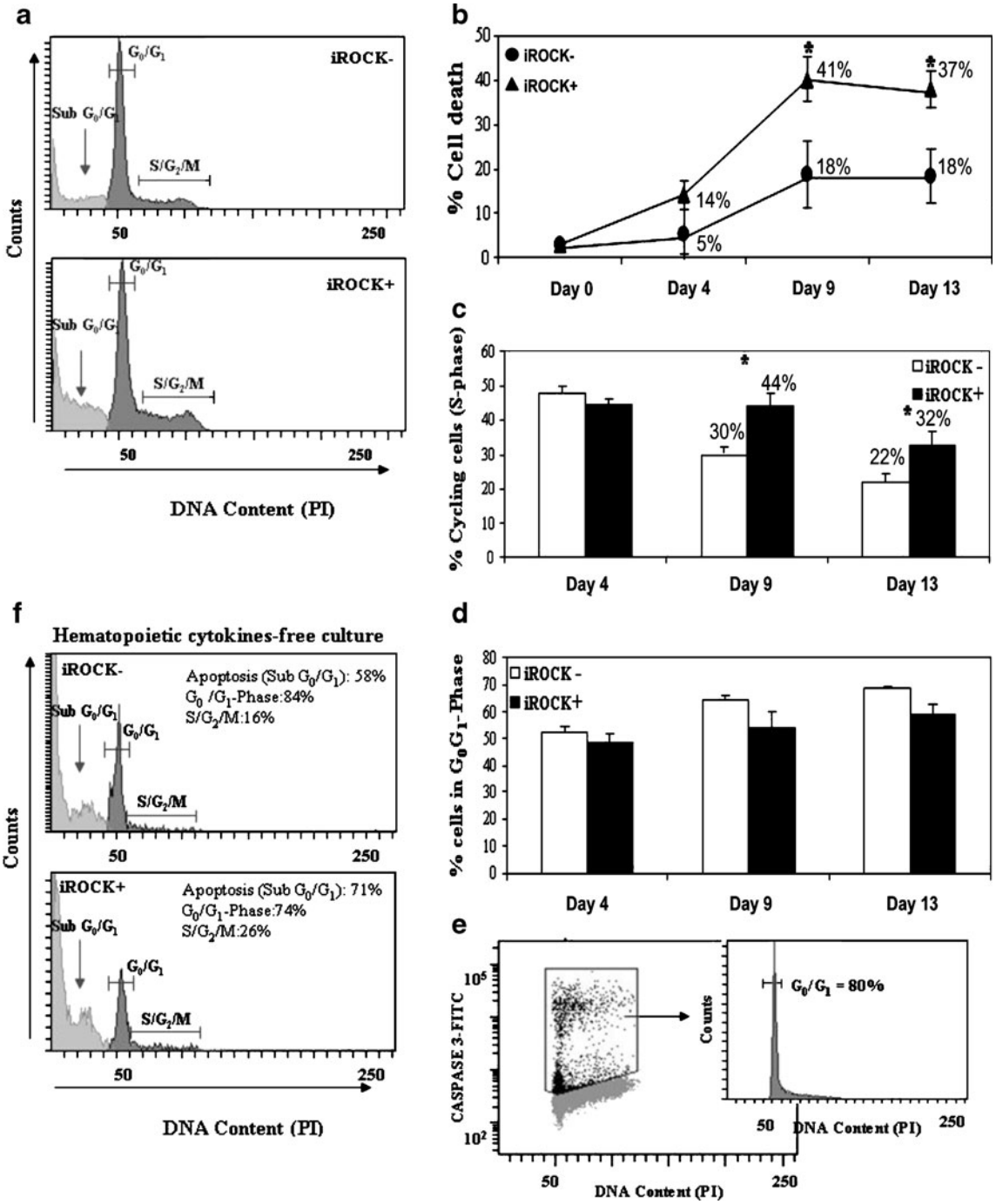

Fig. 2 Y-27632 induces cell death but also cell cycling of CD34+ HSPCs. **a** Representative flow cytometry cell cycle distribution in the presence or absence of the Rock inhibitor Y-27632. **b** Cell death induced by Y-27632 on CD34+ HSPCs measured as Sub G_0/G_1 cell fraction. **c** Proportion of cycling CD34+ cells ($S/G_2/M$) in in vitro cultures with or without Y-27632. **d** Proportion of dormant CD34+ cells (G_0/G_1 phase) in in vitro cultures with or without Y-27632. **e** Representative flow cytometry analysis of active caspase-3 and PI. **f** Representative flow cytometry cell cycle distribution in the presence or absence of the Rock inhibitor Y-27632 under hematopoietic cytokines-free culture conditions. Asterisks represent statistical significant differences ($p<0.05$)

cryopreservation and expansion media also resulted in a strong reduction (7 to 11-fold) of overall cell expansion of frozen-thawed CB-derived cells (Fig. 4a). CD34+ cells cryopreserved in the presence of Y-27632 differentiated normally in vitro, losing CD34 expression within 13 days (Fig. 4b, c). Regarding absolute numbers, Y-27632-supplemented cryopreservation medium and expansion media dramatically inhibited the expansion of CD34+

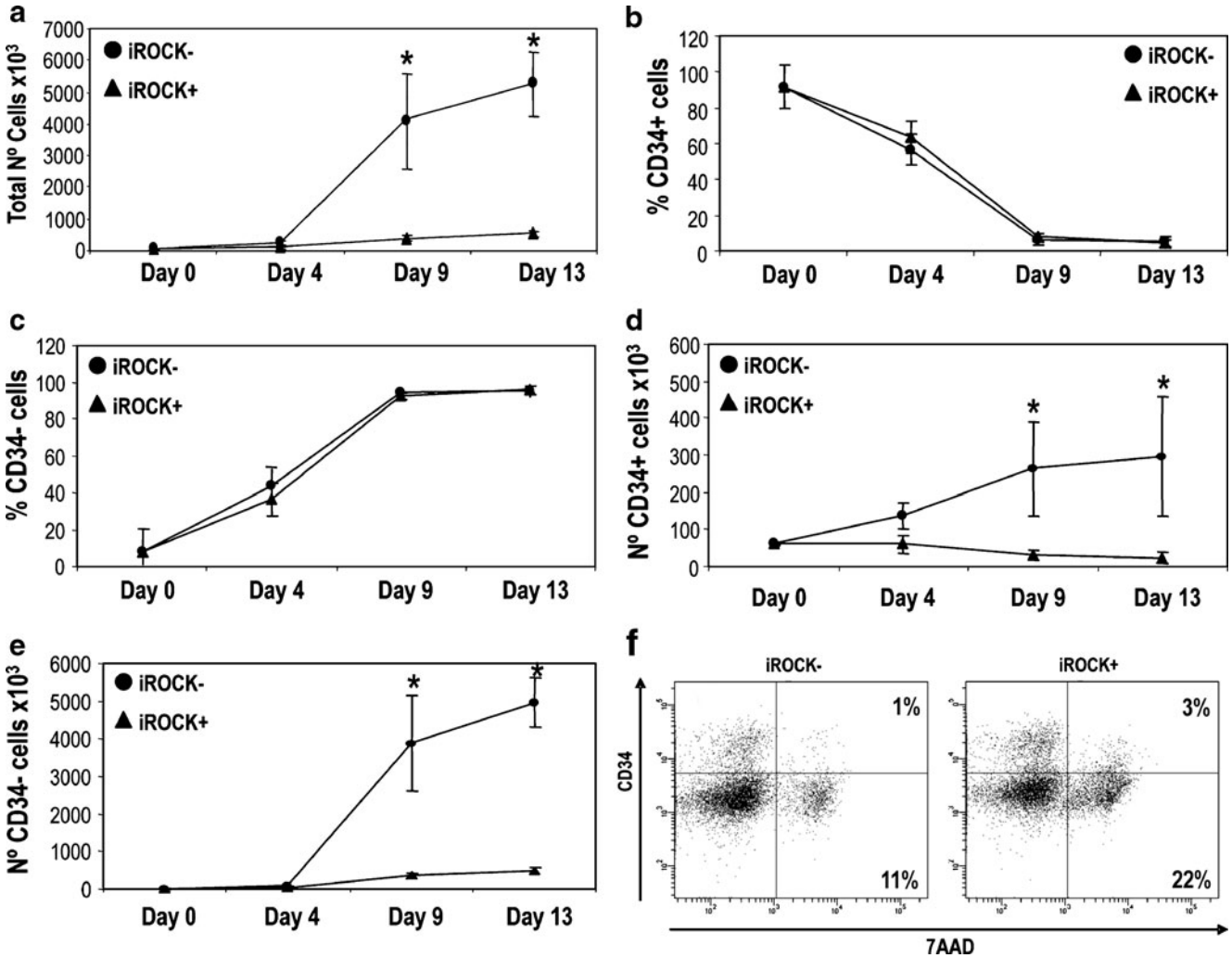

Fig. 3 Y-27632 reduces survival/recovery of cryopreserved CB CD34+ and CD34- cells. **a** Growth curves (cell expansion) of cryopreserved CB-derived CD34+ cells in the presence or absence of Y-27632 measured for up to 13 days. Post-thawing percentage of CD34+ (**b**) and CD34- cells (**c**) at different time points in the presence or absence of Y-27632. Post-thawing absolute numbers of CD34+ (**d**) and CD34- cells (**e**) at different time points in the presence or absence of Y-27632. **f** Representative flow cytometry panels displaying cell death of CD34+ and CD34- cell subsets analysed by 7-AAD staining. Asteriskis represent statistical significant differences ($p<0.05$)

HSPCs (Fig. 4d) and CD34- mature cells (Fig. 4e) throughout the 13-day period. In sum, our results indicate that the ROCK inhibitor Y-27632 not only profoundly inhibits cell expansion of both fresh and cryopreserved CD34+ HSPCs but also improves neither survival nor recovery of CD34+ HSPCs upon thawing regardless Y-27632 being added to both the cryopreservation and the expansion media or just to the expansion medium.

Discussion

The role of HSCT in the treatment of hematologic and non-hematologic malignancies is rapidly expanding. In certain situations fresh HSPCs can be employed in the setting of allogeneic transplantation. However, the current therapeutic strategies demand that the HSPCs are cryopreserved for virtually all autologous and many allogeneic transplants. This strategy has been proven to be safe and not associated with significant adverse outcomes regarding engraftment failure or graft versus host disease [6, 29]. The cryopreservation process is of importance for all types of stem cell collection, but is perhaps particularly critical for CB. The actual transplant is here harvested at the time of birth and used at a later point in time. Despite existing slight variations among transplant centers, the most widely used crypreservation method involves addition of 10% *v/v* DMSO and a constant cooling rate of 1°C per minute. Current methods for ex vivo expansion of HSPCs still result in a loss of multilineage differentiation potential and current freeze-thawing protocols result in significant cell death and loss

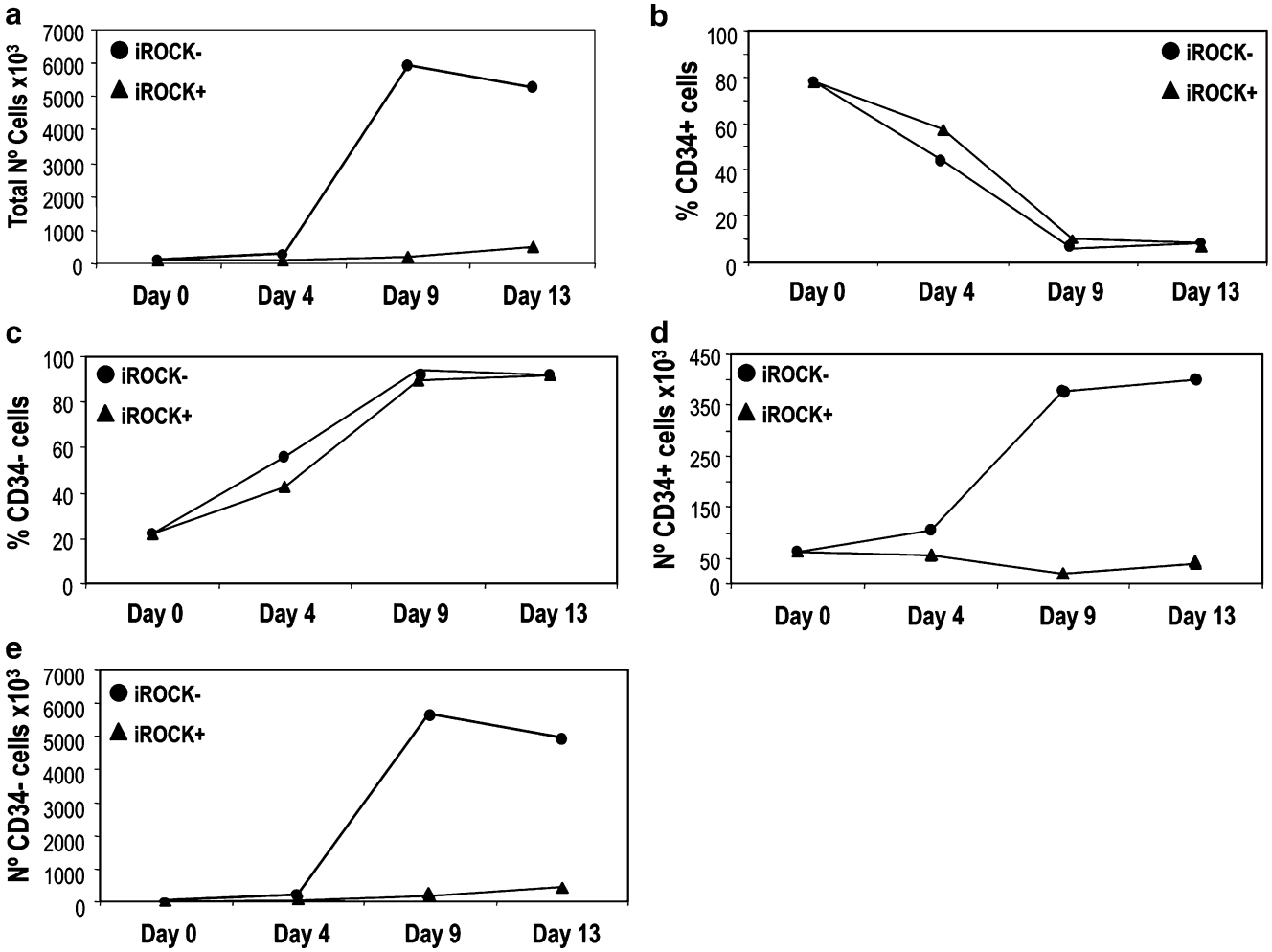

Fig. 4 Supplementation of the cryopreservation medium with Y-27632 does not improve the survival and recovery of thawed CB-derived cells. **a** Y-27632-supplemented cryopreservation medium does not facilitate survival/recovery of thawed CB-derived cells. Post-thawing percentage of CD34+ (**b**) and CD34- cells (**c**) at different time points in the presence or absence of Y-27632 in the expansion media. Post-thawing absolute numbers of CD34+ (**d**) and CD34- cells (**e**) at different time points in the presence or absence of Y-27632 in the expansion media. All the data displayed in Fig. 4 have been generated from CD34+ HSPCs cryopreserved in the presence of Y-27632

of CD34+ HSPCs essential for engraftment, being the apoptosis induced directly by cryoinjury the major cause for the loss of viability after slow freezing.

Very recent reports have demonstrated that Y-27632, a selective and robust inhibitor of p160-Rho-associated coiled-coil kinase (ROCK), is a potent inhibitor of the apoptosis and is efficient in enhancing the post-thaw survival and recovery of frozen human blastocysts [13], hESCs [14–20], iPS cells [21] and hMSCs [22]. Of note, all these human stem cells have in common that they grow attached to the treated tissue culture plastic or extracellular matrix and in many cases they also form physiological cell aggregates. Interestingly, however, the potential effect of Y-27632 on human CB-derived CD34+ HSPCs has not been explored yet. Furthermore, it is worth mentioning that, in contrast to the aforementioned human stem cells (blastocysts, hESCs, iPS cells, MSCs), CD34+ HSPCs grow in suspension rather than attached or as colony aggregates. In the present study, the effect of Y-27632 on culture homeostasis was determined in both fresh and cryopreserved CB-derived CD34+ HSPCs.

Our results indicate that the Y-27632 dramatically inhibits cell expansion of both fresh and cryopreserved CD34+ HSPCs but also improves neither survival nor recovery of CD34+ HSPCs upon thawing regardless whether Y-27632 is added to both the cryopreservation and the expansion media and or just to the expansion medium. These data discourage any clinical application of Y-27632 in potential technical developments aimed at improving cryopreservation procedures of CB-derived cells and/or in vitro expansion of HSPCs without spontaneous differentiation. Our data could be reproduced in the presence and absence of hematopoietic cytokines, suggesting that Y-27632 negatively affects the culture homeostasis

of both fresh and cryopreserved CB-derived CD34+ HSPCs in a cytokine-independent manner.

From a mechanistic standpoint, several recent studies have suggested that hESCs, iPS cells and hMSCs treated with Y-27632 are able to escape apoptosis when dissociated into single cells or detaching them from the treated tissue culture plastic, reinforcing the concept of Y-27632 as a potent inhibitor of apoptosis based upon Rho´s role in accepting signals from G protein-coupled receptors and extracellular matrix (ECM) [30, 31]. In contrast, however, our data on CB-derived CD34+ HSPCs does not support an anti-apoptotic role of the Y-27632. Our data is in line with previous observations indicating that if cell interactions are prevented, Y-27632-treated cells do not survive [15]. Rho kinase and ROCK are ubiquitous proteins which have been found to play a role in regulating multiple cellular processes including cell proliferation, apoptosis, cell-cycle progression, migration, actin cytoskeleton, cell polarity and cell-cell interaction [15, 30]. In contrast to previous reports [12, 13, 16–21], this study identifies for the first time a detrimental effect of Y-27632 on the expansion and survival of both fresh and cryopreserved CB-derived CD34+ HSPCs. Therefore, in agreement with Rancourt´s Lab [15] our data supports that the effect of iROCK on cell-cell interaction and cell adherence may be crucial. We speculate that Y-27632 may have a differential impact not only on distinct lineage/tissue-specific stem cells but may also function differentially on human stem cells growing in suspension versus those growing attached to either treated tissue culture plastic or extracellular matrix. Future work needs to be done to gain further insights into the cellular and molecular mechanisms underlying the apparently differential role of Y-27632 among distinct types of human stem cells.

Acknowledgments This work was funded by the CSJA (0029 and 0030 to P.M) and CICE (P08-CTS-3678 to P.M) de la Junta de Andalucía, The Spanish Ministry of Health to P.M (PI070026) and C. B (CP07/00059) and the MICINN to P.M (PLE-2009-0111).

Authorship C.B: designed and performed experiments, analysed the data and wrote the manuscript. R.M: performed experiments and analysed the data. P.M: conceived and supervised the study and wrote the paper. We thank Dr. René Rodriguez for helpful discussions and Dr Isidro Prat and M.C Rodríguez Lamas for provision of CB Units.

Conflict of Interest Disclosures The authors reported no potential conflicts of interest.

References

1. Gluckman, E., & Rocha, V. (2009). Cord blood transplantation: state of the art. *Haematologica, 94*, 451–454.
2. Levac, K., Menendez, P., & Bhatia, M. (2005). Intra-bone marrow transplantation facilitates pauci-clonal human hematopoietic repopulation of NOD/SCID/beta2m(−/−) mice. *Experimental Hematology, 33*, 1417–1426.
3. Menendez, P., Bueno, C., Wang, L., & Bhatia, M. (2005). Human embryonic stem cells: potential tool for achieving immunotolerance? *Stem Cell Reviews, 1*, 151–158.
4. Menendez, P., Perez-Simon, J. A., Mateos, M. V., Caballero, M. D., Gonzalez, M., et al. (2002). Influence of the different CD34+ and CD34- cell subsets infused on clinical outcome after non-myeloablative allogeneic peripheral blood transplantation from human leucocyte antigen-identical sibling donors. *British Journal Haematology, 119*, 135–143.
5. Menendez, P., Prosper, F., Bueno, C., Arbona, C., San Miguel, J. F., et al. (2001). Sequential analysis of CD34+ and CD34- cell subsets in peripheral blood and leukapheresis products from breast cancer patients mobilized with SCF plus G-CSF and cyclophosphamide. *Leukemia, 15*, 430–439.
6. Berz, D., McCormack, E. M., Winer, E. S., Colvin, G. A., & Quesenberry, P. J. (2007). Cryopreservation of hematopoietic stem cells. *American Journal of Hematology, 82*, 463–472.
7. Bueno, C., Catalina, P., Melen, G. J., Montes, R., Sanchez, L., et al. (2009). Etoposide induces MLL rearrangements and other chromosomal abnormalities in human embryonic stem cells. *Carcinogenesis, 30*, 1628–1637.
8. Bueno, C., Montes, R., De la Cueva, T., Gutierrez-Aranda, I., & Menendez, P. (2009). Intra-bone marrow transplantation of human CD34+ cells into NOD/LtSz-scid IL2rg null mice permits multilineage engraftment without previous irradiation Cytotherapy (in press).
9. Bueno, C., Montes, R., Martin, L., Prat, I., Hernandez, M. C., et al. (2008). NG2 antigen is expressed in CD34+ HPCs and plasmacytoid dendritic cell precursors: is NG2 expression in leukemia dependent on the target cell where leukemogenesis is triggered? *Leukemia, 22*, 1475–1478.
10. Clarke, D. M., Yadock, D. J., Nicoud, I. B., Mathew, A. J., & Heimfeld, S. (2009). Improved post-thaw recovery of peripheral blood stem/progenitor cells using a novel intracellular-like cryopreservation solution. *Cytotherapy, 11*, 472–479.
11. Tijssen, M. R., Woelders, H., de Vries-van, R. A., van der Schoot, C. E., Voermans, C., et al. (2008). Improved postthaw viability and in vitro functionality of peripheral blood hematopoietic progenitor cells after cryopreservation with a theoretically optimized freezing curve. *Transfusion, 48*, 893–901.
12. Heng, B. C., Ye, C. P., Liu, H., Toh, W. S., Rufaihah, A. J., et al. (2006). Loss of viability during freeze-thaw of intact and adherent human embryonic stem cells with conventional slow-cooling protocols is predominantly due to apoptosis rather than cellular necrosis. *Journal of Biomedical Science, 13*, 433–445.
13. Cortes, J. L., Sanchez, L., Ligero, G., Gutierrez-Aranda, I., Catalina, P., et al. (2009). Mesenchymal stem cells facilitate the derivation of human embryonic stem cells from cryopreserved poor-quality embryos. *Human Reproduction, 24*, 1844–1851.
14. Gauthaman, K., Fong, C.-Y., & Bongso, A. (2010). Effect of ROCK Inhibitor Y-27632 on Normal and Variant Human Embryonic Stem Cells (hESCs) In Vitro: Its Benefits in hESC Expansion Stem Cell Rev (in press).
15. Krawetz, R. J., Li, X., & Rancourt, D. E. (2009). Human embryonic stem cells: caught between a ROCK inhibitor and a hard place. *Bioessays, 31*, 336–343.
16. Li, X., Krawetz, R., Liu, S., Meng, G., & Rancourt, D. E. (2009). ROCK inhibitor improves survival of cryopreserved serum/feeder-free single human embryonic stem cells. *Human Reproduction, 24*, 580–589.
17. Li, X., Meng, G., Krawetz, R., Liu, S., & Rancourt, D. E. (2008). The ROCK inhibitor Y-27632 enhances the survival rate of human

embryonic stem cells following cryopreservation. *Stem Cells and Development, 17*, 1079–1085.
18. Martin-Ibanez, R., Unger, C., Stromberg, A., Baker, D., Canals, J. M., et al. (2008). Novel cryopreservation method for dissociated human embryonic stem cells in the presence of a ROCK inhibitor. *Human Reproduction, 23*, 2744–2754.
19. Pakzad, M., Totonchi, M., Taei, A., Seifinejad, A., Hassani, S., et al. (2010). Presence of a ROCK inhibitor in extracellular matrix supports more undifferentiated growth of feeder-free human embryonic and induced pluripotent stem cells upon passaging. Stem Cell Reviews (in press).
20. Watanabe, K., Ueno, M., Kamiya, D., Nishiyama, A., Matsumura, M., et al. (2007). A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nature Biotechnology, 25*, 681–686.
21. Claassen, D. A., Desler, M. M., & Rizzino, A. (2009). ROCK inhibition enhances the recovery and growth of cryopreserved human embryonic stem cells and human induced pluripotent stem cells. *Molecular Reproduction and Development, 76*, 722–732.
22. Heng, B. C. (2009). Effect of Rho-associated kinase (ROCK) inhibitor Y-27632 on the post-thaw viability of cryopreserved human bone marrow-derived mesenchymal stem cells. *Tissue and Cell, 41*, 376–380.
23. Menendez, P., Wang, L., Chadwick, K., Li, L., & Bhatia, M. (2004). Retroviral transduction of hematopoietic cells differentiated from human embryonic stem cell-derived CD45(neg)PFV hemogenic precursors. *Molecular Therapy, 10*, 1109–1120.
24. Catalina, P., Bueno, C., Montes, R., Nieto, A., Ligero, G., et al. (2009). Genetic stability of human embryonic stem cells: a first-step toward the development of hESC-based system for modelling childhhod leukemia. *Leukemia Research, 33*, 980–990.
25. Catalina, P., Montes, R., Ligero, G., Sanchez, L., de la Cueva, T., et al. (2008). Human ESCs predisposition to karyotypic instability: is a matter of culture adaptation or differential vulnerability among hESC lines due to inherent properties? *Molecular Cancer, 7*, 76–81.
26. Wang, L., Menendez, P., Cerdan, C., & Bhatia, M. (2005). Hematopoietic development from human embryonic stem cell lines. *Experimental Hematology, 33*, 987–996.
27. Menendez, P., Caballero, M. D., Prosper, F., Del Canizo, M. C., Perez-Simon, J. A., et al. (2002). The composition of leukapheresis products impacts on the hematopoietic recovery after autologous transplantation independently of the mobilization regimen. *Transfusion, 42*, 1159–1172.
28. Prosper, F., Sola, C., Hornedo, J., Arbona, C., Menendez, P., et al. (2003). Mobilization of peripheral blood progenitor cells with a combination of cyclophosphamide, r-metHuSCF and filgrastim in patients with breast cancer previously treated with chemotherapy. *Leukemia, 17*, 437–441.
29. Stockschlader, M., Hassan, H. T., Krog, C., Kruger, W., Loliger, C., et al. (1997). Long-term follow-up of leukaemia patients after related cryopreserved allogeneic bone marrow transplantation. *British Journal Haematology, 96*, 382–386.
30. Hall, A. (1994). Small GTP-binding proteins and the regulation of the actin cytoskeleton. *Annual Review of Cell Biology, 10*, 31–54.
31. Koyanagi, M., Takahashi, J., Arakawa, Y., Doi, D., Fukuda, H., et al. (2008). Inhibition of the Rho/ROCK pathway reduces apoptosis during transplantation of embryonic stem cell-derived neural precursors. *Journal of Neuroscience Research, 86*, 270–280.