Cryobiology xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

In situ cryopreservation of human embryonic stem cells in gas-permeable membrane culture cassettes for high post-thaw yield and good manufacturing practice ☆

K.J. Amps *, M. Jones, D. Baker, H.D. Moore

Centre for Stem Cell Biology, Department of Biomedical Science, University of Sheffield, Western Bank, Sheffield S10 2TN, UK

ARTICLE INFO

Article history:
Received 16 October 2009
Accepted 18 March 2010
Available online xxxx

Keywords:
Human embryonic stem cell
Gas permeable membrane
Cryopreservation *in situ*
Relative efficiency

ABSTRACT

The development of efficient and robust methods for the cryopreservation of human embryonic stem cells (hESCs) is important for the production of master and working cell banks for future clinical applications. Such methods must meet requirements of good manufacturing practice (GMP) and maintain genetic stability of the cell line. We investigated the culture of four Shef hESC lines in gas permeable 'culture cassettes' which met GMP compliance. hESCs adhered rapidly to the membrane and colonies displayed good proliferation and expansion. After 5–7 days of culture, hESCs were cryopreserved *in situ* using 10% dimethyl sulphoxide in foetal calf serum at ~1 °C/min. This method was compared with a control of standard flask culture and cryopreservation in vials. Post-thaw cassette culture displayed relative proliferation ratios (fold increase above flask/cryovial culture) of 114 (Shef 4), 8.2 (Shef 5), 195 (shef 6) and 17.5 (Shef 7). The proportion of cells expressing pluripotency markers after cryopreservation was consistently greater in cassette culture than for the control with the markers SSEA3 and SSEA4 exhibiting a significant increase ($P \geqslant 0.05$). The efficiency of cell line culture in cassette was associated with the overall passage number of the cell line. The procedure enables cryopreservation of relatively large quantities of hESCs *in situ*, whilst returning high yields of viable, undifferentiated stem cells, thereby increasing capacity to scale up with greater efficacy.

Introduction

Human embryonic stem cells (hESCs) are pluripotent cells derived from the pre-implantation embryo [20]. Stable cell lines generated with these cells have apparently unlimited proliferative capacity and differentiate into many cell types of the body [5,19]. Because of these attributes, there is great interest in using hESCs in therapies to treat degenerative diseases, so-called regenerative medicine. The clinical use of hESCs demands the need for all the processes within production to meet the requirements of the medical regulatory agency(s) of a country or region (i.e. USA—Federal Drug Administration; EU—European Medicines Agency) and this usually involves the implementation of good manufacturing practice (GMP). Such hESC lines should have a full and traceable history with cell lines demonstrating a stable plating efficiency, survival and proliferative capacity after cryopreservation [8]. Therefore the development of efficient and robust systems for the cryopreservation of hESCs for the production of seed (master) and working cell banks is of utmost importance for any future clinical applications.

hESCs are relatively sensitive to conditions of cryopreservation perhaps reflecting their origin in the early pre-implantation human embryo which also requires exacting conditions to survive freezing and thawing [14,15]. After a standard equilibrium 'slow' freezing procedure using 10% dimethylsulphoxide (Me_2SO, DMSO) in fetal calf serum (FCS) as cryoprotectant it is estimated that less than 5% of hESCs survive the cryopreservation and thawing process [17]. By contrast, 'vitrification' methods using an 'open pulled straw' procedure enable high viability of hESCs (~80%) after thawing, but only with very small volumes (1–5 μl) thereby limiting the number of cells cryopreserved at any one time. Moreover, vitrification requires the operator to transfer the cell suspension between cryoprotectant solutions with precise timing to minimise toxic effects and thus the efficacy of freezing (cell viability, batch variation) is greatly influenced by operator experience and training. Vitrification tends to be the method of choice for cells shortly after being derived, transfected or cloned when cell numbers are low but there is an urgent need for preservation as insurance against subsequent culture failure. Despite its inefficiency, equilibrium freezing remains the preferred method for routine freezing of bulk amounts of cells. While both techniques are in routine use in many

☆ This study was funded by a grant from the Medical Research Council, UK.
* Corresponding author.
E-mail address: k.amps@shef.ac.uk (K.J. Amps).

0011-2240/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.cryobiol.2010.03.007

research laboratories, they entail a long post-thaw amplification of cell numbers from a single aliquot and neither method is satisfactory for GMP [8,9].

Besides the immediate effects of protection from cryo-injury, the overall efficiency of hESC recovery after cryopreservation is determined by several other factors. The ultimate endpoint for a cryopreserved aliquot of stem cells is a proliferating, pluripotent culture of hESC colonies for expansion of cells to subsequently generate the cell of choice. Therefore, the rates of cell differentiation and apoptosis after thawing also have a major impact on recovery and these factors vary between cell lines. hESCs are maintained in a stem cell niche created in culture as the colony develops, either in the presence of feeder cells (usually mitotically inactivated mouse or human embryonic fibroblasts), or more recently with extracellular matrix components (e.g. Matrigel) and defined culture medium [16]. Cryoprotective agents (CPAs) such as Me_2SO and ethylene glycol can cause rapid cellular and irreversible differentiation to the niche such that very few pluripotent cells remain to renew the culture and hence these chemicals must be quickly and effectively removed after thawing. Of equal significance, hESCs will rapidly undergo apoptosis or differentiation when dispersed to single cells or very small colonies when they are recovered from their monolayer adherent culture [1,9] prior to cryopreservation in suspension and following thawing and being initially returned to culture. For this reason methods have been devised to protect the stem cell niche and cryopreserve hESCs *in situ* within culture plate wells and flasks and such procedures have been shown to be considerably more effective than more conventional cell suspension methods [12].

Finally, the efficiency of cryopreservation of hESCs is clearly of practical importance in both the research and clinical setting. However, it is paramount for seed and working cell banks used for the clinic. Poor cryopreservation may contribute to genetic mutation in the cell line which if not detected could ultimately result in serious long-term consequences for the patient. The stresses imposed on the cell during a cryopreservation and thawing cycle are very considerable and a genetic change that gives a selective advantage to a cell to cope with this process (adaptation) may be favoured and rapidly spread through the culture [2,7]. Hence it is imperative to minimise this risk with cryogenic procedures that reduce any selection on the cell line.

Here we describe a cryopreservation system for hESCs involving their culture and cryopreservation in gas permeable 'culture cassettes' which are suitable for GMP requirements and bridges the gap between the outcomes of equilibrium and vitrification freezing. The procedure enables cryopreservation of relatively large quantities of hESCs *in situ*, whilst returning high yields of viable, undifferentiated stem cells, with the capacity to scale up culture with greater efficacy.

Materials and methods

Media and chemicals

All media and supplements were from Invitrogen (Paisley, UK) and other chemicals from Sigma–Aldrich (Poole, Dorset, UK) unless otherwise stated.

hESC lines and maintenance

Shef 4, Shef 5, Shef 6, Shef 7 hESC lines used in the study were generated by the Centre for Stem Cell Biology (CSCB) under license from the Human Fertilisation and Embryology Authority (HFEA) according to fully approved ethical guidelines. These hESC lines have been verified and deposited in the UK Stem Cell Bank for international distribution. Shef cell lines display marker profiles of pluripotency and differentiation [1,10]. All stock cultures of hESC lines were chromosomally normal at the beginning of the study as tested by karyotype analysis using the G-banding technique [6]. Cells were maintained as described previously under standard protocols at 37 °C at 5% CO_2 in air [5]. Briefly, hESCs were maintained as colonies in culture with mitomycin C inactivated mouse embryonic fibroblasts (MEFs, MF-1 strain) seeded at passage 5 at a density of 1×10^4 cells per cm^2 on pre-gelatinised (0.1% gelatine solution) T25 flasks (NUNC, Fisher Scientific, Leicester, UK) with hESC medium consisting of KODMEM medium supplemented with 20% Knockout Serum Replacement, 1% non-essential amino acids (PAA), 1 mM L-glutamine; 0.1 mM β-mercapto-ethanol and 4 ng/ml human basic fibroblast growth factor (bFGF) (Peprotech EC Ltd., London, UK). Cells were passaged enzymatically using either collagenase IV (1 mg/ml) or accutase (1 mg/ml) treatment usually every 4–5 days when colony density reached ~80% confluence [5]. Seeding density was kept comparable between flasks by splitting colcultures evenly between treatments.

Cell culture in cassettes

MEF feeders were seeded onto one side of a 10 ml Clinicell cell culture cassette (Mabio International, France) using a similar method to T25 flask culture with 2.5×10^4/ml per cassette transferred by 5 ml sterile plastic syringe (BD Biosciences) using the Luer connection according to the manufacturer's instructions. The cassette was then topped up with fibroblast medium with all air eliminated and laid on its side in an incubator at 37 °C at 5% CO_2 in air for cell attachment to one membrane surface. hESCs were transferred to the cassette with feeders after 1–3 days by recovery from a parent flask using collagenase, centrifuged at 300*g* for 5 min and resuspended in 11 ml of hESC medium. Fibroblast medium was emptied from the cassette and the hESC suspension transferred by syringe transfer with the cassette fully topped up without air bubbles. The cassette was laid on its side in the incubator for hESCs to attach to the feeder layer. Culture medium was changed every 2–3 days for fresh medium using syringe transfer. Cells in the cassette were subjected to *in situ* freezing when hESC colonies were confluent on day 4–7 days of passage depending on the cell line. For further passage to assess cell proliferation after cryopreservation hESCs were harvested from cassettes using accutase at a 1:3 dilution for 8 min before tapping the cassette to release the cells, the suspension was then recovered by syringe, centrifuged at 300*g* for 5 min, resuspended in hESC medium and passaged (1:2) as described above. Controls were subjected to similar passage conditions.

hESC cryopreservation

hESC suspension from a stock flask was allocated to cassette or T25 flask culture (*in situ* control) as described above or subjected directly to a standard equilibrium 'slow freezing' ('harvest' control). For the latter, the cell suspension was centrifuged at 300*g* for 3 min and the pellet resuspended in 1 ml freezing medium at 4 °C consisting of 10% Me_2SO in FCS in a 2 ml cryovial (Fisher Scientific, Leicester, UK) placed in a Nalgene isopropanol container (Mr Frosty, Sigma–Aldrich) and transferred to a −80 °C freezer for −1 °C/min cooling rate. After 24 h the vials were transferred directly to liquid nitrogen in a storage dewar. hESC cultures in cassettes or flasks were considered ready for cryopreservation when colony size was maximal but morphological indications of cell differentiation were still minimal. hESC medium was removed and replaced with chilled (4 °C) freezing medium to cover the cells and placed flat in a polysterene box in the −80 °C freezer. Preliminary experiments were performed on various box sizes to enable

Please cite this article in press as: K.J. Amps et al., *In situ* cryopreservation of human embryonic stem cells in gas-permeable membrane culture cassettes for high post-thaw yield and good manufacturing practice, Cryobiology (2010), doi:10.1016/j.cryobiol.2010.03.007

approximate slow cooling conditions. Some flasks and cassettes were transferred to liquid nitrogen in a dewar for 1 h to simulate −196 °C storage conditions. These preparations were not bag sealed to prevent potential liquid nitrogen ingress and the hazard of gas pressure build-up on thawing, although this sort of problem did not occur in our experiments.

Cell thawing

Aliquots of hESCs cryopreserved by standard slow freezing were thawed in the routine manner of the laboratory. A cryovial of hESCs (harvest control) was held by hand in a 37 °C water bath and swirled around with the top out of water until the suspension was thawed. The top of the vial was wiped with 70% alcohol and the suspension transferred to a centrifuged tube, slowly diluted with 10 ml of hESC medium and centrifuged at 300*g* for 5 min. The cell pellet was resuspended in 5 ml of hESC medium and transferred to T25 flask culture with MEF feeders. For *in situ* cryopreservation, the culture cassette or flask was held by hand in the 37 °C water bath keeping the Luer ports or top clear of water and swirling around until the contents had thawed. CPA medium was removed and replaced with hESC medium. This was performed two times to remove traces of CPA. The cassette or flask was then transferred to an incubator at 37 °C at 5% CO_2 in air. hESC number was amplified by further passages (2–8 depending on the cell line) for flow cytometry analysis. A relative cell proliferation ratio was determined as follows:

$$[\text{cell concentration in cassette}/\text{cell concentration in flask}] \times \text{split fraction} \times \text{passage number}.$$

Determination of pluripotency markers by immunocytochemistry and flow cytometry (FACS)

hESCs were recovered as described above and washed by centrifugation with 5% foetal calf serum in PBS, and treated with trypsin/EDTA for 3 min to form single cells. The suspension was inactivated with 10% FCS in DMEM and cells washed twice by centrifugation at 300*g* for 3 min and live cell number (trypan blue exclusion) determined by haemocytometer before antibody labelling. Briefly, aliquots of single cells were incubated for 30 min with monoclonal antibodies (hybridoma supernatants) to embryonic stem cell markers, SSEA3, SSEA4, Tra-1-60 and SSEA1; the pan human marker Tra-1-85 (CD 147, basigin); and control myeloma supernatant P3X. The cells were washed three times by centrifugation and incubated with goat anti-mouse IgG + IgM (Caltag) for a further 30 min, Following washing by centrifugation, cell were labelled with propidium iodide and flow cytometry (Cyan, Beckman Coulter) performed as described previously [4].

Chromosomal analysis

Post-thaw cultures were examined by fluorescent *in situ* hybridisation (FISH) by analysing 20 cells per culture [6].

Results

Experiments were carried out in triplicate for each cell line with cassette culture treatment and controls.

Cassette culture of hESCs

The addition of culture medium and transfer of cells at passage by syringe rather than pipette was straightforward (Fig. 1A). There was maximal adherence of feeder cells to the cassette polycarbonate membrane with little cell loss. After initial passage hESCs adhered rapidly and established colonies with good proliferative growth which were comparable or exceeded conventional culture in flasks (Fig. 1B and C). In general, hESC colonies remained undifferentiated for a longer period in cassette culture (7–10 days) compared with flasks (4–6 days) and therefore cassettes generated greater numbers (×2–5) of hESCs per cm^2 with colonies close together.

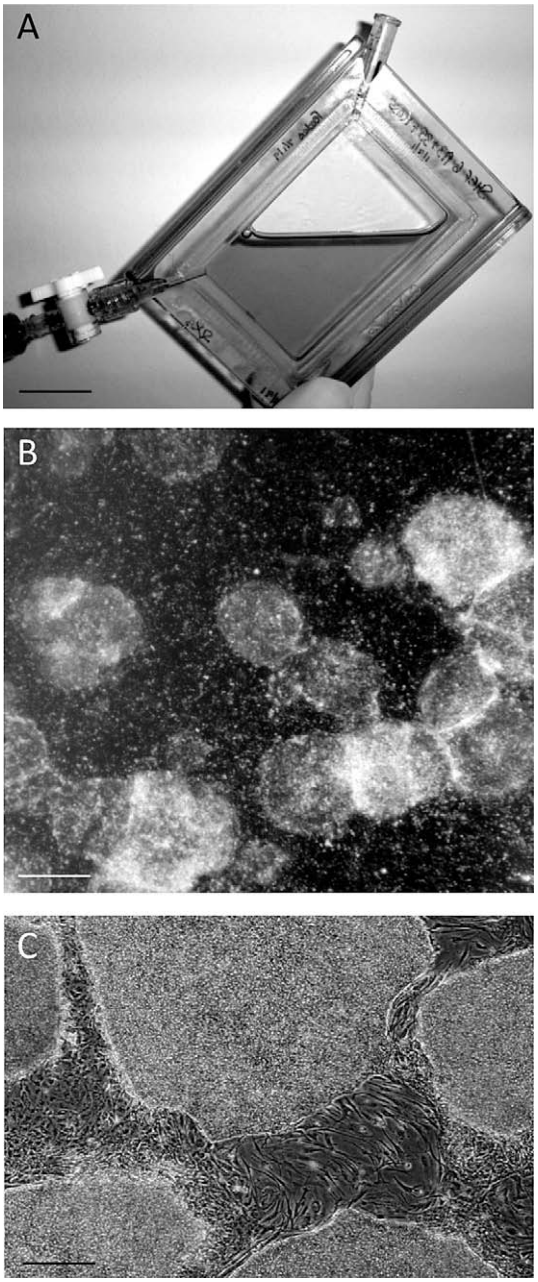

Fig. 1. Cassette culture of hESCs (Shef 5). (A) Clinicell culture cassette being filled with hESC cell suspension showing general arrangement and procedure for transferring cells. Bar = 2 cm; (B) dark field view of hESC colonies after four days of culture in cassette. Bar = 200 μm; (C) At higher magnification, hESC colonies and feeder cells on day prior to cryopreservation. Bar = 50 μm.

Cryopreservation of hESCs and subsequent culture

Cryopreservation of hESCs *in situ* in flasks was poor and unreliable for all the cell lines with retention and recovery of very few hESC colonies (0–3 per flask) after post-thaw culture for 5 days.

Please cite this article in press as: K.J. Amps et al., *In situ* cryopreservation of human embryonic stem cells in gas-permeable membrane culture cassettes for high post-thaw yield and good manufacturing practice, Cryobiology (2010), doi:10.1016/j.cryobiol.2010.03.007

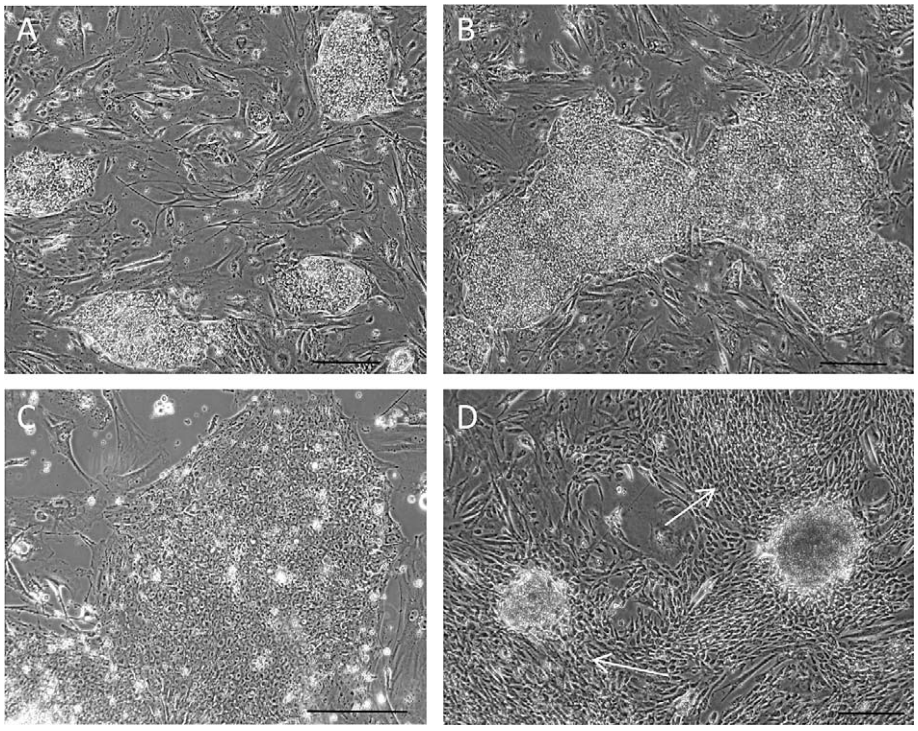

Fig. 2. Micrographs of cells in cassette culture (A-C) or in flask control. (A) Typical well-adhered hESC colony formation 2 days post-thaw with good feeder cell morphology; (B) HESC colony expansion and consolidation after 3 days post-thaw; (C) higher magnification of colony 3 days post-thaw showing good hESC morphology; (D) Typical hESC colonies after control 'harvest' cryopreservation and flask culture 4 days post-thaw. Note differentiated cell proliferation (arrowed) and lack of colony spreading. Bars = 20 μm.

This technique could not be used as a practical control and therefore the 'harvest' control of a hESC aliquot (from same cell line and batch) for standard equilibrium freezing in cryovials and subsequent culture by standard flask procedures was used for comparison with cassette *in situ* cryopreservation, thawing and culture. Many hESC colonies (>100) retained adherence and viability in cassettes following freezing/thawing with clear indications of colony proliferation within 24–48 h post-thaw (Fig. 2A–C). By comparison, the 'harvest' control post-thaw cultures with equivalent cell numbers initially generated just a few attached colonies (2–8 per flask) which were often much smaller. Although more colonies appeared after several days post-thaw (10–20 flask), presumably from small hESC aggregates or single cells, these were slow to proliferate and exhibited a great degree of differentiation. For each cell line the proportion of cells expressing pluripotency markers was consistently greater in cassette culture than for the flask control after cryopreservation (Fig. 3). For the mean values for all cell lines, there was a significant increase in the proportion of hESCs cells expressing SSEA3 and SSEA4 in cassette culture compared to flask control culture post-thaw (Fig. 4).

There was relatively much greater number of hESCs generated in cassette culture than in the control for all the cell lines (Table 1). Mean relative efficiency of cassette culture compared to flask control as determined by the cell number ratio varied considerably. For Shef 5 and 7 this was 8.2 and 17.5, respectively while Shef 4 and 6 exhibited much greater ratios of 195 and 115, respectively.

All cell lines cultured in cassette or flask were karyotypically normal after cryopreservation except for Shef 5 where 1 of 20 cells analysed from control flask culture displayed an abnormal karyoptype.

Discussion

The aim of this empirical study was to develop a method for rapid amplification of hESC number after cryopreservation using methods which would be effective and suitable for production of master (seed) and working cell banks of clinical grade cell lines. The overall efficiency of hESC cryopreservation is important to maximise the use of cell lines while minimising the risk of genetic mutations. Equilibrium 'slow' freezing methods promote apoptosis and spontaneous differentiation of hESCs cells. On the other hand, vitrification protocols are much more efficient but can only be used with very small volumes. Cultures of Shef 4–7 hESCs cell lines were cryopreserved using a standard equilibrium freezing method using cryovials (harvest method), or directly *in situ* in culture cassettes (Clinicell). Subsequently these cells were thawed and further passaged for analysis of cell pluripotency and differentiation, and cell counts and relative efficiencies of the two methods calculated and compared. A relative proliferation ratio was used as an integrated endpoint measure of the overall cell survival, proliferation and pluripotent capacity. This is because with hESCs, the post-thaw generation of pluripotent cell colonies and their expansion rate is much more important than merely an immediate cell viability measurement as apoptosis and differentiation are significant factors. Moreover, it can be determined with minimal disruption of the cultures. However, this method limits information about single cell survival immediately post-thaw,

The results indicate that for a comparable number of cells *in situ* cryopreservation in cassette culture substantially improved post-thaw cultures of hESCs compared to cryopreservation in vials and flasks without affecting the gross genetic stability (as measured by karyotype) of the cell lines. Many more stem cell colonies were established and underwent undifferentiated expansion post-thaw in cassettes than in flasks following standard cryovial cryopreservation and passage. Previous studies of hESCs cryopreserved on extracellular matrix (Matrigel) in wells of culture plates have also demonstrated better cell survival and subsequent cell proliferation after equilibrium freezing [12]. The present study used feeder cells (which produce their own matrix) rather than Matrigel but the

Please cite this article in press as: K.J. Amps et al., *In situ* cryopreservation of human embryonic stem cells in gas-permeable membrane culture cassettes for high post-thaw yield and good manufacturing practice, Cryobiology (2010), doi:10.1016/j.cryobiol.2010.03.007

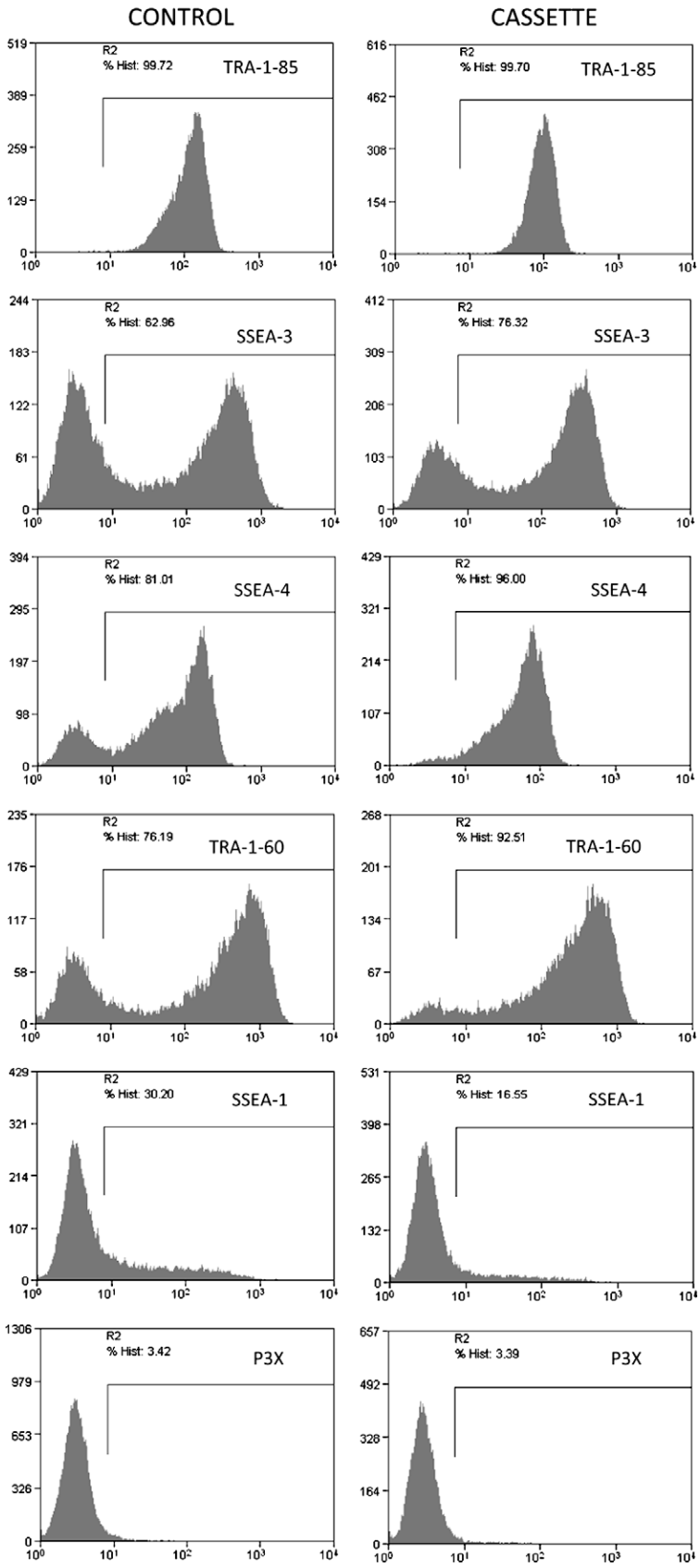

Fig. 3. Post-thaw FACS analysis of pluripotency/differentiation expression for Shef 6 hESCs following control 'harvest' or cassette cryopreservation. Tra-1-85 (pan-human marker, basigin); SSEA3, SSEA4 and Tra-1-60 pluripotency markers; SSEA1 differentiation marker; P3X antibody control. Similar results were obtained for all the cell lines.

outcome was similar. If hESCs are disaggregated from their pluripotent stem cell niche, which involves a colony of cells adhered to a surface matrix, they undergo rapid apoptosis and differentiation. Thus, the efficiency of establishing new colonies after cell passage

Please cite this article in press as: K.J. Amps et al., *In situ* cryopreservation of human embryonic stem cells in gas-permeable membrane culture cassettes for high post-thaw yield and good manufacturing practice, Cryobiology (2010), doi:10.1016/j.cryobiol.2010.03.007

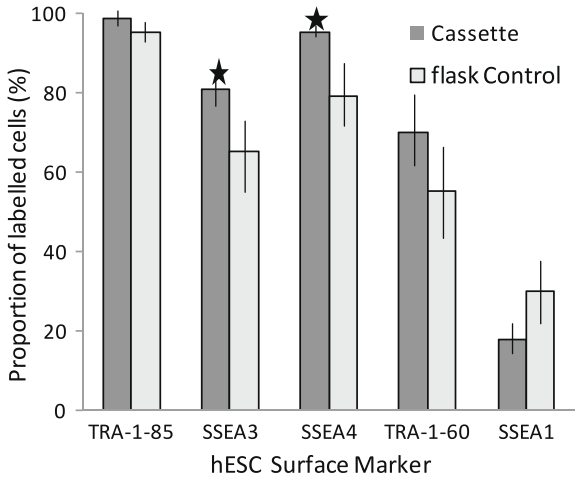

Fig. 4. Histogram of the mean proportion of labelled hESCs for various markers after post-thaw culture following cassette or flask 'harvest' control cryopreservation ($n = 10$). Significantly different from control $P \leqslant 0.05$. Bar = ±standard deviation.

Table 1
Post-thaw proliferation rates for Shef hESC lines after cryovial or cassette freezing.

ShefhESC line (passage #)	Post-thaw cell proliferation (10^6 cells)		Proliferation ratio
	Cryovial/flask	Cassette	
4 (90)	3.2 ± 1.6	624 ± 82**	195
5 (30)	1.2 ± 0.3	21.8 ± 3.6*	17.5
6 (63)	2.4 ± 0.9	265 ± 31**	115
7 (37)	1.5 ± 0.4	12.4 ± 2.4*	8.2

* Significantly different $P \leqslant 0.05$.
** Significantly different $P \leqslant 0.001$. $n = 3$ for each treatment.

is relatively low for clumps of hESCs and extremely low when cells are single (0.005%). When other factors are equal, then procedures for cryopreservation that involve hESC disaggregation are clearly at a disadvantage. The greater early survival and then expansion of hESC colonies post-thaw in cassette culture appeared to be critical for achieving rapid proliferation of pluripotent cells and led up to a >200-fold relative increase in pluripotent hESCs compared to using cryovial and routine passaging in flasks over a similar time period. However, there were significant differences between cell lines in post-thaw proliferation. This was greater than the regular variation in proliferation parameters between cell lines in routine culture suggesting that the response to *in situ* cryopreservation is to some extent dependent on the cell line.

Several aspects of a hESC line will contribute to the survival and proliferation of cells after cryopreservation. While most hESC cell lines in general display similar gene expression patterns when maintained in culture as undifferentiated or differentiating cells [10], differences are also apparent which probably reflect the genetic composition of the source embryo. Therefore, these differences may be reflected in the overall ability to withstand the various stresses of freezing and thawing and the capacity to form and maintain a proliferative stem cell colony. Genetic differences in the capacity for cryopreservation have been reported for mouse embryonic stem cells [11,18] although data with hESCs is mainly ancedotal. However, hESC lines may also undergo changes after prolonged passage number as cells are preferentially selected with an advantage for survival under the particular culture conditions—so called 'adaptation' [2,7]. Overall Shef 4 and 6 lines had undergone more passages (90 and 63, respectively) than Shef 5 and 7 lines (30 and 37, respectively) prior to the study. This passage difference correlated with significant disparity in the relative post-thaw proliferation of cells in cassettes (Table 1) perhaps indicating an adaptation process for cells to adhere in culture. It is of interest that this difference manifested with the cassette culture and cryopreservation rather than standard cryopreservation procedures.

Cassette culture had several advantages for processing clinical grade hESCs. The system is a medical device (complying with the European Directive 93/42/EEC) intended for therapeutic use and cells can be processed in a 'closed' manner, thereby minimising risk of contamination with adventitious agents. hESC colonies also expanded for longer periods than in flasks before requiring passage due to differentiation. The reason for this is unclear but may relate to the good gas exchange across the semi-permeable membrane of the cassette or perhaps better adherence of cells to membrane rather than the flask plastic. The longer time between passages was a significant factor in the expansion of hESC cell number post-thaw because, as mentioned above the disruption of colonies at passage compromises cell proliferation.

A number of investigations are now in progress to improve *in situ* hESC cryopreservation and culture. Recent reports indicate that Rho-associated kinase inhibitor (ROCK) improves post-thaw survival and proliferation of hESCs in suspension by preventing their apoptosis [3,13]. Therefore if cryopreservation causes disruption to the adherence of colonies *in situ*, this can be minimised with supplementation of CPA and culture medium with such inhibitors. The culture of hESCs in cassettes under feeder-free conditions using defined medium [16] may also allow further expansion of cells prior to passage to maximise cell numbers. Finally, systematic investigation of the immediate post-thaw survival of hESCs on membrane may establish the optimal cooling curve for freeze-thawing cassette which will further improve cell survival.

Acknowledgments

This study was supported by a grant from the MRC. We are grateful for excellent technical assistance from Heather Spink, Gemma Bray and Gregg Bingham. We thank Dominique Malard of Laboratories MABIO International for advice on Clinicell Cassettes.

References

[1] B. Aflatoonian, L. Ruban, S. Shamsuddin, D. Baker, P. Andrews, H. Moore, Generation of Sheffield (Shef) human embryonic stem cell lines using a microdrop culture system. In Vitro Cell Dev. Biol. Anim. (2010) Mar 12. [Epub ahead of print].
[2] D.E. Baker, N.J. Harrison, E. Maltby, K. Smith, H.D. Moore, P.J. Shaw, P.R. Heath, H. Holden, P.W. Andrews, Adaptation to culture of human embryonic stem cells and oncogenesis in vivo, Nat. Biotechnol. 25 (2007) 207–215.
[3] D.A. Claassen, M.M. Desler, A. Rizzino, ROCK inhibition enhances the recovery and growth of cryopreserved human embryonic stem cells and human induced pluripotent stem cells, Mol. Reprod. Dev. 76 (2009) 722–732.
[4] J.S. Draper, C. Pigott, J.A. Thomson, P.W. Andrews, Surface antigens of human embryonic stem cells: changes upon differentiation in culture, J. Anat. 200 (2002) 249–258.
[5] J.S. Draper, H.D. Moore, L.N. Ruban, P.J. Gokhale, P.W. Andrews, Culture and characterization of human embryonic stem cells, Stem Cells Dev. 13 (2004) 325–336.
[6] C. Hanson, G. Caisander, Human embryonic stem cells and chromosome stability, APMIS 113 (2005) 751–755.
[7] N.J. Harrison, D. Baker, P.W. Andrews, Culture adaptation of embryonic stem cells echoes germ cell malignancy, Int. J. Androl. 30 (2007) 275–281.
[8] Z.A. Hewitt, K.J. Amps, H.D. Moore, Derivation of GMP raw materials for use in regenerative medicine: hESC-based therapies, progress toward clinical application, Clin. Pharmacol. Ther. 82 (2007) 448–452.
[9] C.J. Hunt, P.M. Timmons, Cryopreservation of human embryonic stem cell lines, Methods Mol. Biol. 368 (2007) 261–270.
[10] International Stem Cell Initiative O. Adewumi et al., Characterization of human embryonic stem cell lines by the International Stem Cell Initiative, Nat. Biotechnol. 25 (2007) 803–816.
[11] C.M. Kashuba Benson, J.D. Benson, J.K. Critser, An improved cryopreservation method for a mouse embryonic stem cell line, Cryobiology 56 (2008) 120–130.
[12] L. Ji, J.J. de Pablo, S.P. Palecek, Cryopreservation of adherent human embryonic stem cells, Biotechnol. Bioeng. 88 (2004) 299–312.

[13] X. Li, G. Meng, R. Krawetz, S. Liu, D.E. Rancourt, The ROCK inhibitor Y-27632 enhances the survival rate of human embryonic stem cells following cryopreservation, Stem Cells Dev. 17 (2008) 1079–1085.
[14] Y. Li, J.C. Tan, L.S. Li, Comparison of three methods for cryopreservation of human embryonic stem cells, Fertil. Steril. 93 (2010) 999–1005.
[15] J. Liebermann, J. Dietl, P. Vanderzwalmen, M.J. Tucker, Recent developments in human oocyte, embryo and blastocyst vitrification: where are we now?, Reprod Biomed. Online 7 (2003) 623–633.
[16] T.E. Ludwig, V. Bergendahl, M.E. Levenstein, J. Yu, M.D. Probasco, J.A. Thomson, Feeder-independent culture of human embryonic stem cells, Nat. Methods 3 (2006) 637–646.
[17] B.E. Reubinoff, M.F. Pera, G. Vajta, A.O. Trounson, Effective cryopreservation of human embryonic stem cells by the open pulled straw vitrification method, Hum. Reprod. 16 (2001) 2187–2194.
[18] E.J. Robertson, Embryo derived stem cell lines, in: E.J. Robertson (Ed.), Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, IRL Press, Oxford, 1987, pp. 71–112.
[19] J. Silva, A. Smith, Capturing pluripotency, Cell 132 (2008) 532–536.
[20] J.A. Thomson, J. Itskovitz-Eldor, S.S. Shapiro, M.A. Waknitz, J.J. Swiergiel, V.S. Marshall, J.M. Jones, Embryonic stem cell lines derived from human blastocysts, Science 282 (1998) 1145–1147.

Please cite this article in press as: K.J. Amps et al., *In situ* cryopreservation of human embryonic stem cells in gas-permeable membrane culture cassettes for high post-thaw yield and good manufacturing practice, Cryobiology (2010), doi:10.1016/j.cryobiol.2010.03.007