Human Reproduction, Vol.24, No.8 pp. 1844–1851, 2009
Advanced Access publication on April 28, 2009 doi:10.1093/humrep/dep107

human reproduction

ORIGINAL ARTICLE *Embryology*

Mesenchymal stem cells facilitate the derivation of human embryonic stem cells from cryopreserved poor-quality embryos

J.L. Cortes[1], L. Sanchez[1], G. Ligero[1], I. Gutierrez-Aranda[1], P. Catalina[1], C. Elosua[1], P.E. Leone[1], R. Montes[1], C. Bueno[1], V. Ramos-Mejía[1], I. Maleno[2], J.L. García-Pérez[1], and P. Menendez[1,3]

[1]Andalusian Stem Cell Bank (BACM), Centro de Investigación Biomédica, CSJA-UGR, Parque Tecnológico de la Salud, Avda del Conocimiento s/n, Granada, Spain [2]Servicio de Análisis Clínicos, Hospital Virgen de las Nieves, Granada, Spain

[3]Correspondence address. Tel: +34-958-894672; Fax: +34-958-894652; E-mail: pablo.menendez@juntadeandalucia.es

BACKGROUND: Human embryonic stem cells (hESCs) have opened up a new area of research in biomedicine. The efficiency of hESC derivation from frozen poor-quality embryos is low and normally achieved by plating embryos on mouse or human foreskin feeders (HFFs). We attempted to optimize embryo survival and hESC derivation.

METHODS: Three conditions were tested on frozen poor-quality embryos: (i) embryo treatment with the Rho-associated kinase (ROCK) inhibitor, Y-27632; (ii) use of human mesenchymal stem cells (hMSCs) as feeders; and (iii) laser drilling (LD) for inner cell mass (ICM) isolation. Two hundred and nineteen thawed embryos were randomly treated with ($n = 110$) or without ($n = 109$) 10 μM Y-27632. Surviving embryos that developed to blastocyst stage ($n = 50$) were randomly co-cultured on HFFs ($n = 21$) or hMSCs ($n = 29$). ICM isolation was either by whole-blastocyst culture (WBC) or WBC plus LD.

RESULTS: Embryo survival was 52% higher with Y-27632. hMSCs appeared to facilitate ICM outgrowth and hESC derivation: three hESC lines were derived on hMSCs (10.3% efficiency) whereas no hESC line was derived on HFFs. ROCK inhibition and ICM isolation method did not affect hESC efficiency. The lines derived on hMSCs (AND-1, -2, -3) were characterized and showed typical hESC morphology, euploidy, surface marker and transcription factor expression and multilineage developmental potential. The hESC lines have been stable for over 38 passages on hMSCs.

CONCLUSION: Our data suggest that Y-27632 increases post-thaw embryo survival and that hMSCs may facilitate the efficiency of hESC derivation from frozen poor-quality embryos.

Key words: human embryonic stem cell derivation / human mesenchymal stem cells / ROCK inhibitor / Y-27632 / laser drilling

Introduction

Human embryonic stem cell (hESC) research represents a nascent area of investigation. hESCs are pluripotent stem cells derived from the inner cell mass (ICM) of human blastocyst-stage embryos (Thomson *et al*., 1998; Reubinoff *et al*., 2000) and are defined by both robust self-renewal capacity and pluripotent developmental potential *in vitro* and *in vivo* (Menendez *et al*., 2006). Accordingly, cultures of hESCs represent a theoretically inexhaustible source of pluripotent cells that can be differentiated into any cell type and, therefore, have been hailed as a unique tool in a range of biomedical studies, such as cell replacement therapy, developmental biology, drug discovery and disease modelling (Menendez *et al*., 2005; Bueno *et al*., 2008a, b).

The blastocyst quality, ICM isolation method used and the culture conditions (feeders) employed are likely the three main factors dictating either ESC derivation failure or success (Cortes *et al*., 2007a; Cortes and Menendez, 2008). Regarding blastocyst quality, in many countries worldwide only cryopreserved human embryos created during IVF which are in excess of clinical need or deemed clinically useless based on poor morphology may be used for hESC derivation (Cortes *et al*., 2007b). These frozen supernumerary human embryos are usually of poor-quality (Lerou *et al*., 2008). Theoptimal methods to isolate the ICM are still controversial. The ICM is usually isolated

from the expanded blastocyst using a variety of techniques, including immunosurgery (Solter and Knowles, 1975), mechanical processes (Bongso *et al*., 1994) and whole blastocyst culture (WBC) (Kim *et al*., 2005). These methods are associated with some challenges, including the use of xeno-components, which may prevent the use of hESC derivatives in potential future therapeutic applications, and the low ESC establishment efficiency (Cortes and Menendez, 2008). Recently, we and others have developed a laser-assisted system for isolation of the ICM from mouse and human embryos (Tanaka *et al*., 2006; Cortes *et al*., 2007a; Cortes and Menendez, 2008; Turetsky *et al*., 2008). In addition, the derivation and maintenance of hESCs normally require the use of feeder cells (Amit *et al*., 2003). Up until now, either mouse foreskin feeders (MEFs), STO fibroblast (Park *et al*., 2004) or human foreskin feeders (HFFs) (Hovatta *et al*., 2003) were used as support for hESC line derivation. Although MEFs and HFFs are the most commonly used feeders for hESC co-culture, we and others have recently shown successful hESC maintenance in human mesenchymal stem cells (hMSCs) or hMSC-conditioned media (Cheng *et al*., 2003; Montes *et al*., 2009). In line with the mesoderm origin of hMSCs, we hypothesize that hESC lines derived on hMSCs may be more prone to differentiation toward mesodermal lineages (Ledran *et al*., 2008). Thus, the optimization of hESC line derivation methods is challenging and it is still unclear which approach is the most efficient (Hovatta, 2006).

Human ESCs are susceptible to apoptosis upon cell detachment and dissociation (Watanabe *et al*., 2007). The selective Rho-associated kinase (ROCK) inhibitor Y-27632 has been reported to increase the survival of dissociated hESCs and their cloning efficiency and to improve their survival upon cryopreservation (Watanabe *et al*., 2007; Martin-Ibañez *et al*., 2008; Li *et al*., 2008, 2009). However, whether treatment of cryopreserved human embryos with the ROCK inhibitor Y-27632 facilitates embryo survival and augments the efficiency of hESC establishment still needs to be examined.

In the present study, we attempted to optimize the hESC derivation process from frozen poor-quality embryos. Three experimental conditions were tested for improving embryo survival and hESC derivation efficiency: (i) post-thaw embryo treatment with the selective ROCK inhibitor Y-27632; (ii) the use of hMSCs as feeders and, (iii) laser drilling (LD) technology for ICM isolation. Our data suggest that hMSCs increase the efficiency of hESC derivation from frozen poor-quality embryos. In contrast, although treatment with Y-27632 increased embryo survival, neither ROCK inhibition nor the ICM isolation method used seems to enhance the efficiency of hESC establishment.

Materials and Methods

Cryopreserved human embryos

This study was approved by our Local Authorities and the Spanish National Embryo Steering Committee. Cryopreserved human embryos ($n = 219$) were donated to this study upon informed consent by couples that had already undergone an IVF cycle (Cortes *et al*., 2007b). These human embryos were frozen between 1995 and 2004.

As shown in our experimental design (Fig. 1), the embryos were initially thawed in the presence or absence of 10 μM of ROCK inhibitor Y-27632 (Sigma, St Louis, MO, USA) (Narumiya *et al*., 2000), which has been previously reported to increase the survival of single clones of hESCs, their cloning efficiency, and their survival upon cryopreservation (Watanabe *et al*., 2007; Martin-Ibañez *et al*., 2008; Li *et al*., 2008, 2009). Y-27632 was added to the human embryo culture media, G-1 v.5 and G-2 v.5 (Vitrolife, Sweden).

Embryo outgrowth co-culture on allogeneic human feeders

Human embryos which survived and developed up to the blastocyst stage were cultured in four-well plates (BD Labware, Franklin Lakes, NJ, USA) either on a confluent layer of γ-inactivated (4000 rads) HFFs (Hovatta *et al*., 2003) or hMSCs (Schäffler and Büchler, 2007; Uccelli *et al*., 2008; Garcia-Castro *et al*., 2008). HFFs were purchased from The American Type Culture Collection (SCD-1112SK). hMSCs were obtained from postnatal adipose tissue from healthy donors upon informed consent as previously described (Cobo *et al*., 2008; Garcia-Castro *et al*., 2008). HFFs and hMSCs were grown in Iscove's Modified Dulbecco's Media and advanced Dulbecco's modified Eagle's medium (DMEM), respectively, supplemented with 10% fetal calf serum and 2 mM L-glutamine. The four-well plates were seeded with 0.5×10^5 cells/cm^2 HFFs or hMSCs. Successful embryo outgrowths were routinely maintained in medium consisting of 80% KO-DMEM supplemented with 20% KO Serum Replacement, 1% non-essential amino acids, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol and 8 ng/ml of basic fibroblast growth factor (all from Invitrogen, CA, USA), independent of the allogeneic human feeders.

ICM isolation methods

Two different experimental approaches were used for ICM isolation and further hESC line establishment depending on the quality of the blastocyst: WBC method alone or WBC assisted by laser (WBC + LD) (Cortes and Menendez, 2008): the latter ICM isolation method has been previously described (Cortes and Menendez, 2008). WBC assisted by LD was employed for blastocysts with a large and distinguishable ICM, whereas WBC alone was used for those blastocysts with a tiny or indistinguishable ICM (Kim *et al*., 2005; Cortes *et al*., 2007a; Cortes and Menendez, 2008). Briefly, when WBC was used, the zona pellucida was removed by Tyrodés Acid (Irvine Scientific, CA, USA) for no more than 1 min. Then, the whole blastocyst was cultured in such a way that the trophoectoderm cells and the ICM cells adhered to the feeders (Fig. 2A–D). Subsequently, the distinguishable ICM was carefully plucked and transferred to a freshly prepared human feeder layer and allowed to expand.

Those blastocysts treated with WBC + LD were initially treated with Tyrodés Acid for no more than 1 min, to ensure the complete dissolution of zona pellucida, and allow the trophoectoderm cells and ICM to adhere to the feeder layer. By day 3 of culture the trophoectoderm cells began to expand leaving the ICM accessible, forming a dome-like structure (Cortes and Menendez, 2008). At this point, the trophectoderm cells were laser-shot, leaving the ICM free of trophoectoderm cells and reducing the risk of dragging the trophectoderm cells when the ICM was plucked and transferred to a freshly prepared human feeder layer (Cortes and Menendez, 2008) (Fig. 2A–E).

Characterization of established hESCs

Established hESCs were characterized by indirect immunocytochemistry using antibodies against stage-specific embryonic antigen (SSEA)-3, SSEA-4 (Developmental Studies Hybridoma Bank, University of Iowa, USA), TRA-1-60 and TRA-1-81 (Chemicon, CA, USA). Briefly, hESC colonies were cultured in chamber slides. Cells were fixed in 4% paraformaldehyde for 20 min followed by 30 min incubation in 10% normal goat serum in phosphate-buffered saline (PBS). Colonies were incubated with primary antibodies (1:100 dilution in PBS) for 1 h at room temperature (RT). A fluorescein isothiocyanate (FITC)-conjugated anti-mouse

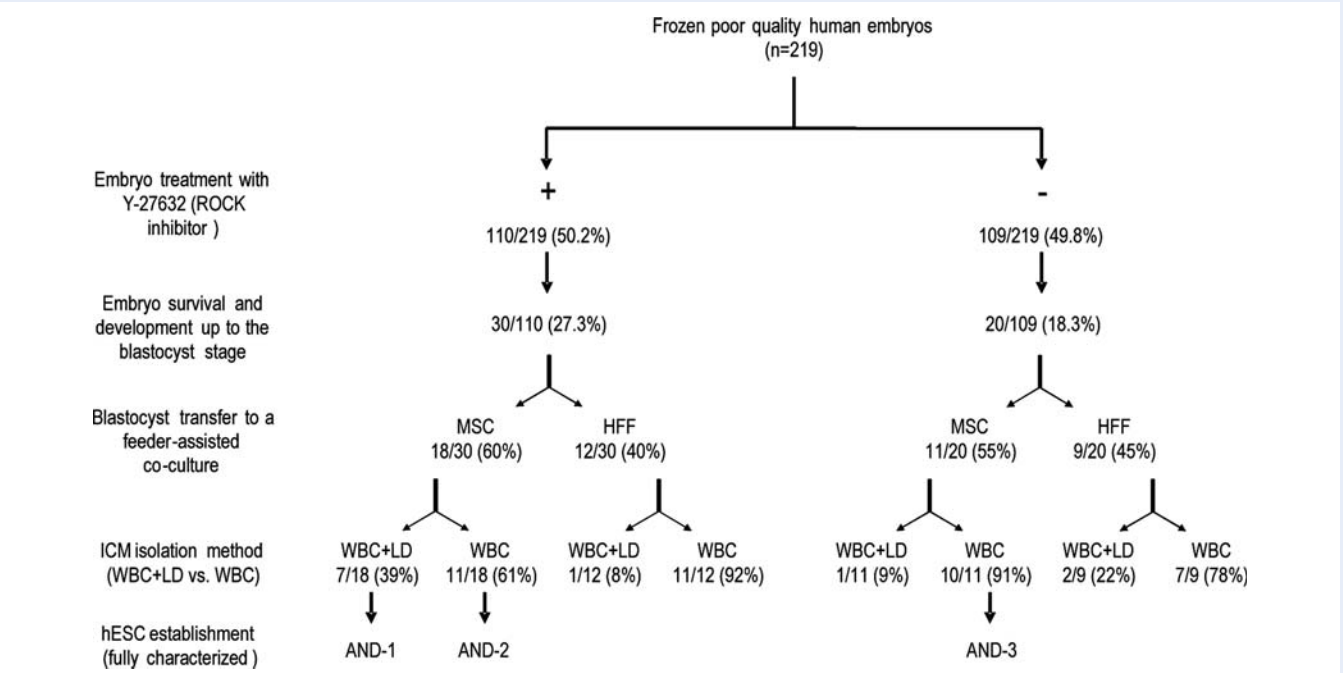

Figure 1 Experimental strategy used for the derivation of hESC lines from poor-quality frozen embryos using hMSCs.

Two hundred and nineteen frozen poor-quality human embryos were used. Embryos were randomly treated with ($n = 110$) or without ($n = 109$) 10 μM ROCK inhibitor, Y-27632. Surviving embryos which reached the blastocyst stage were transferred to a feeder layer of hMSCs or HFFs to ascertain which of these allogenic human feeders better support the expansion of the ICM and subsequent establishment of hESC lines. To isolate a large and easily identifiable ICM we used WBC followed by LD while for blastocysts with a very small or indistinguishable ICM, WBC was used. Three hESC lines were established and fully characterized (see Figs. 3–5 and Supplementary Figure 1). hESC, human embryonic stem cell; hMSC, human mesenchymal stem cell; ROCK Rho – associated kinase; HFF, human forestin feeders; WBC, whole blastocyst culture; LD, laser drilling; ICM, inner cell mass.

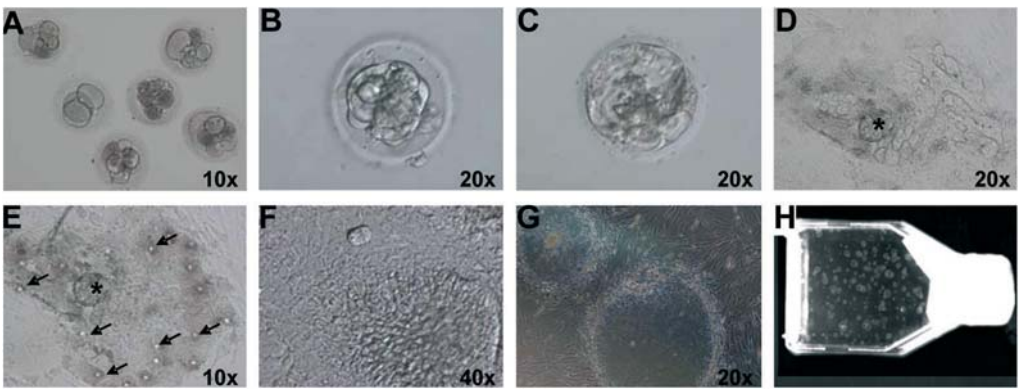

Figure 2 The hESC derivation process from frozen-thawed human embryos.

Representative examples of the process are shown. (**A**) Day-3 poor-quality embryos immediately after thawing. (**B**) Blastocyst in the cavitations stage. (**C**) Poor-quality expanded blastocyst. (**D**) Outgrowth of blastocyst adhered to hMSCs 3 days after ICM isolation (WBC technique). The asterisk indicates the ICM. (**E**) Outgrowth of a representative blastocyst adhered to hMSCs 3 days after isolation (WBC + LD). The asterisk shows the ICM. The black arrows depict the exact laser shots (white dots). (**F**) Replating of the expanded ICM onto hMSCs at passage 1. (**G**) Typical morphology of a hESC colony growing on hMSCs after >30 passages. (**H**) A phase-contrast image of T25-flask showing multiple hESC colonies growing on MSCs after >30 passages. hESC, human embryonic stem cell; hMSC, human mesenchymal stem cell; ICM, inner cell mass.

immunoglobulin (Ig)M secondary antibody (1:100 dilution in PBS) was used for 30 min at RT (Jackson Laboratories Inc.). The slides were mounted in Vectashield containing 4′,6-diamidino-2-phenylindole (Vector Laboratories Inc.). As a negative control, the primary antibodies were replaced by PBS. The same markers were assessed by flow cytometry using an irrelevant isotype-match. Trypsin-dissociated hESC lines were suspended in PBS + 3% fetal bovine serum (FBS) at a concentration of $2–5 \times 10^4$ cells per 100 μl and incubated with the specific primary

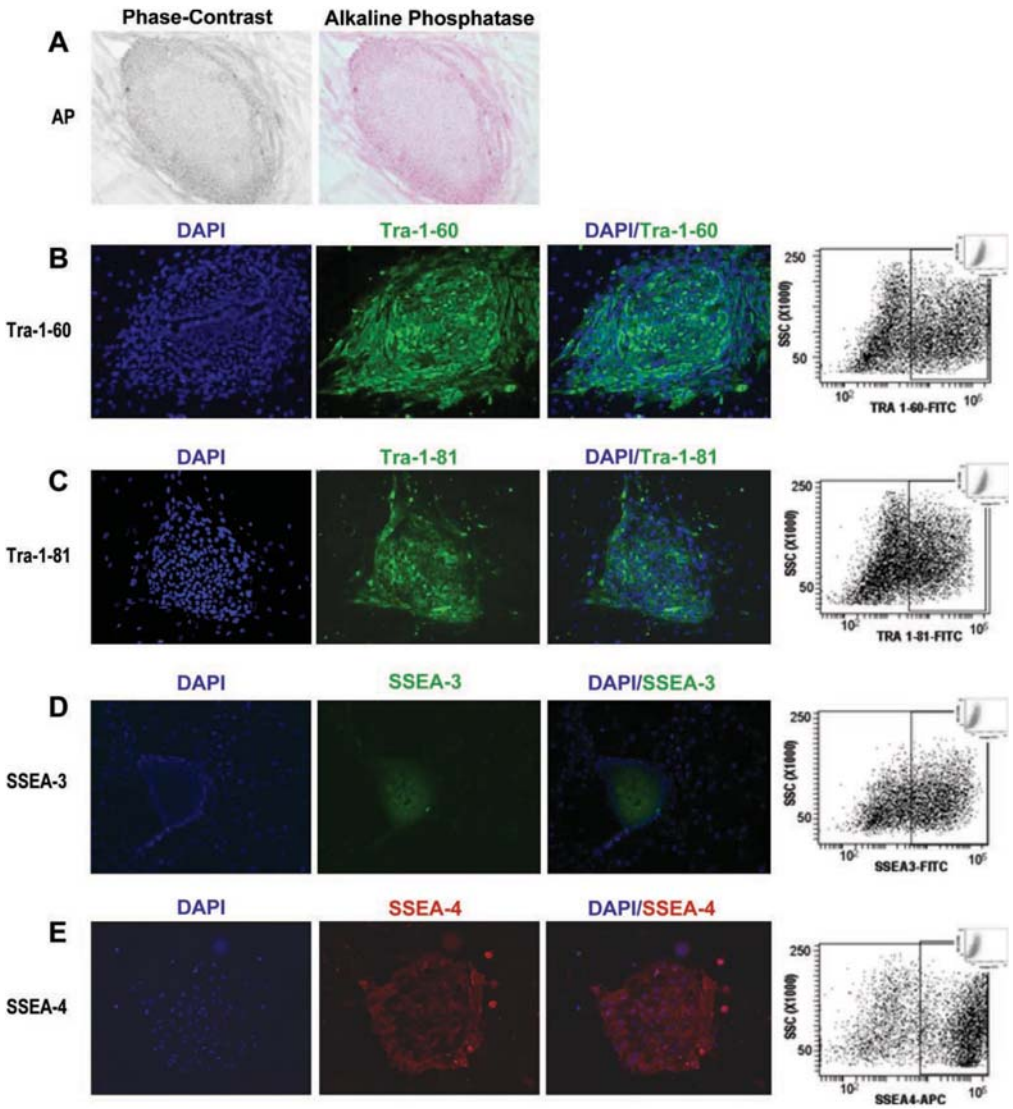

Figure 3 Phenotypic characterization of the hESCs derived on hMSCs.

(**A**) Phase-contrast image (left panel) and representative alkaline phosphatase (AP, right panel) staining. (**B**) Immunocytochemistry staining against Tra-1-60, (**C**) Tra-1-81, (**D**) stage-specific embryonic antigen (SSEA)-3 and (**E**) SSEA-4. Nuclei were counterstained with 4,6-diamino-2-phenylindole (DAPI, blue). The far right panels confirm the expression of Tra-1-60, Tra-1-81, SSEA-3 and SSEA-4 by flow cytometry. Isotypes are shown as insets. hESC, human embryonic stem cell; hMSC, human mesenchymal stem cell.

antibody for 30 min at 4°C. After washing with PBS + 3%FBS, cells were incubated with 2.5 μl of FITC-conjugated goat anti-mouse IgG antibody (Immunotech, Marseille, France). After 15 min at RT, the cells were washed in PBS + 3%FBS and finally stained with 7-aminoactinomycin D (7-AAD) (Immunotech) for 5 min at RT. Live cells identified by 7-AAD exclusion were analyzed for expression of SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81 using a FACSCanto-II flow cytometer equipped with the FACSDiva software (BDB) (Bueno *et al*., 2008a, b). Alkaline phosphatase (AP) expression was demonstrated using the AP Detection Kit (Chemicon).

Molecular and cytogenetic analysis

The expression of the pluripotency-associated transcription factors Oct3/4, Nanog, Rex-1 and Sox2 were assessed by end-point RT–PCR (Catalina *et al*., 2008a). Glyceraldehyde-3-phosphate dehydrogenase was used as a housekeeping gene control. The following PCR conditions were used: 5 min at 94°C, 35 cycles of 30 s at 94°C followed by 50 s at 60°C and 50 s at 72°C and a final extension of 10 min at 72°C, and products were resolved in 1% agarose gel (Catalina *et al*., 2008a).

Conventional karyotyping analysis was performed as previously described in detail and 20–30 metaphase spreads per hESC line were analyzed (Menendez *et al*., 2005; Catalina *et al*., 2008a, b).

In vitro differentiation analysis

Near confluent hESCs were treated with collagenase IV for 5 min at 37°C, transferred (2×10^2 cells/cm^2) to non-adherent plates and allowed to differentiate spontaneously by embryoid body (EB) formation in DMEM supplemented with 20% FBS, 1% L-glutamine, 0.1 mM non-essential amino acids and 0.1 mM β-mercaptoethanol with media changes every 4 days. After 21 days of EB differentiation, EBs were spun down, fixed

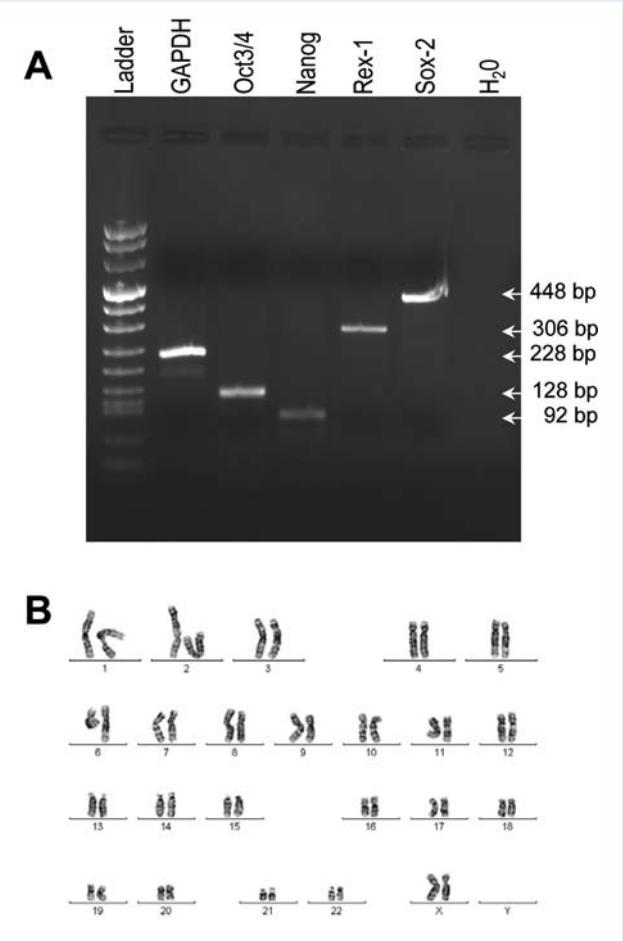

Figure 4 Molecular and cytogenetic characterization of the hESCs derived on hMSCs.

(**A**) RT–PCR analysis showing expression of Oct 3/4, Nanog, Rex1 and Sox2 in undifferentiated hESC cultures. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. (**B**) A representative G-banding analysis depicting euploid karyotype. hESC, human embryonic stem cell; hMSC, human mesenchymal stem cell.

with 4% paraformaldehyde for 10 min and embedded in paraffin (Catalina *et al*., 2008a). For each staining, three sections per specimen were used. Sections were incubated (1 h at RT) with the primary antibodies anti-α-fetoprotein (Santa Cruz Biotechnology; 1:500 dilution in PBS), anti-β-III Tubulin (Chemicon, 1:100 dilution in PBS) and anti-smooth-muscle actin (Chemicon, 1:100 dilution in PBS). Slides were then incubated with a biotinylated secondary antibody (30 min at RT) and a streptavidin peroxidase complex (30 min at RT) (both from Vector Laboratories Inc.). The immunostaining was visualized using diaminobenzidine and counterstained with hematoxylin. All the washing steps used PBS.

In vivo teratoma formation in NOD/SCID-IL2Rγ$^{-/-}$ mice

During routine passage, 20–50 clumps consisting of about 100 undifferentiated cells each were harvested and injected into the testis of 6- to 8-week-old nonobese diabetic/severely compromised immunodeficient (NOD/SCID)IL2Rγ$^{-/-}$ mice. Eight to ten weeks later, the resulting teratomas were fixed in 10% neutral-buffered formalin, embedded in paraffin, and examined histologically after hematoxylin and eosin staining as previously described (Catalina *et al*., 2008a; Cortes and Menendez, 2008).

Short tandem repeat typing

Short tandem repeat (STR) analysis was carried out using the Geneprint® Fluorescent STR Multiplex-290 GammaSTR® kit (Promega, Madison, WI, USA). ElectropheroANDm data were collected with the ABI PRISM 3100 DNA Sequencer (Applied Biosystems), and analyzed using the Genotyper®3.7 software (Applied Biosystems) as previously described (Catalina *et al*., 2008a).

Results

Y-27632 rock inhibitor increases cryopreserved embryo survival

As shown in our experimental approach (Fig. 1), 219 poor-quality human embryos which had been cryopreserved between 1995 and 2004 were thawed to establish hESC lines. Embryos were randomly treated with ($n = 110$) or without ($n = 109$) 10 μM Y-27632. Treatment with Y-27632 induced a 52% increase in embryo survival: 30 surviving embryos out of 110 thawed embryos (27.3%) and 20 surviving embryos out of 109 thawed embryos (18%) in the presence and absence of Y-27632, respectively. Thus, Y-27632 treatment increased the survival of thawed embryos.

hMSCs may facilitate the derivation of hESC lines from frozen poor-quality embryos

We investigated the effect of two distinct human feeder layers: hMSCs versus HFFs, and two different ICM isolation methods, depending on the embryo quality. Surviving embryos which reached the blastocyst stage were randomly transferred to a feeder layer composed of hMSCs or HFFs to ascertain which of these allogenic human feeders better support the expansion of the ICM and subsequent establishment of hESC lines. Of the 50 embryos which survived the cryopreservation procedure and reached the blastocyst stage (Fig. 2A–C), 29 were plated on hMSCs and 21 on HFFs (Fig. 1). The ICM was subsequently isolated using either WBC ($n = 39$, Fig. 2D) or laser-assisted WBC ($n = 11$, Fig. 2E), depending on the blastocyst quality. ICM outgrowths (Fig. 2F) were allowed to expand. Expanded ICMs were subsequently transferred to freshly prepared feeders and allowed to grow further to give rise to hESC lines. hMSCs used as feeders facilitated ICM outgrowth and hESC derivation: three hESC lines could be derived on hMSCs (3 hESC lines out of 29 blastocysts; 10.3% efficiency) whereas no hESC line could be derived on HFFs (0 hESC lines out of 21 blastocysts; 0% efficiency) (Fig. 1). However, neither ROCK inhibition nor the ICM isolation method seemed to affect efficiency of hESC establishment (Fig. 1). The three hESC lines derived on hMSCs, termed AND-1, -2, -3, were fully characterized. The STR typing profile (Supplementary Fig. 1) clearly differs among AND-1, AND-2 and AND-3, confirming the unique identity of these independently derived hESC lines and lack of cross-contamination. Taken together, our data suggest that hMSCs increase the efficiency of hESC derivation from frozen poor-quality embryos whereas neither ROCK inhibition nor the ICM isolation method seems to enhance the efficiency of hESC establishment.

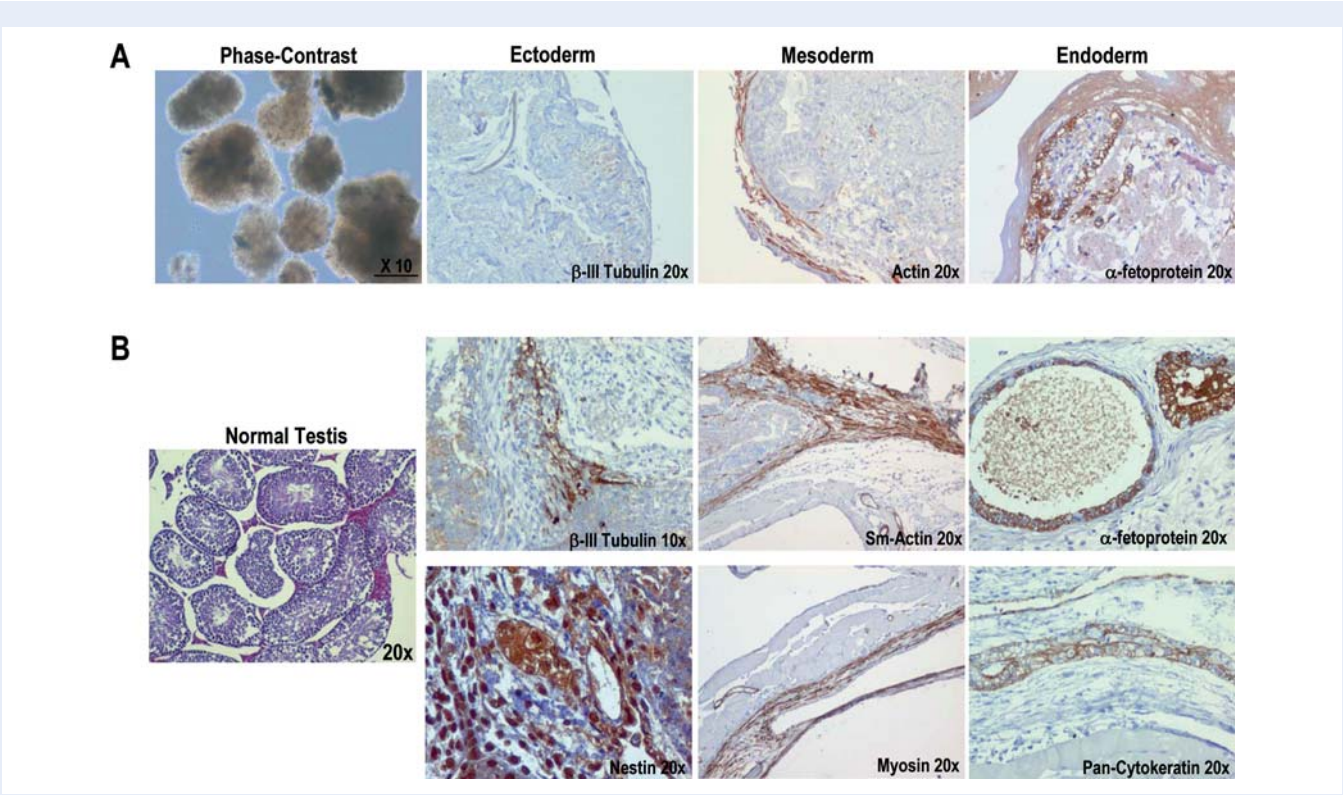

Figure 5 hESC lines derived on hMSCs and maintained on hMSCs or in hMSC-conditioned medium retain potential for three germ layer differentiation.

(**A**) Histological analysis of EBs showing spontaneous *in vitro* differentiation into ectoderm (β-III Tubulin positive), mesoderm (Actin positive) and endoderm (α-fetoprotein positive). (**B**) Teratomas developed in mice 8–10 weeks after inoculation of hESCs under the testicular capsule and histological studies revealed the presence of tissues representing the three germ layers: ectoderm, mesoderm and endoderm. Representative image (left) of a control mouse testicle, not injected with hESCs, showing normal seminiferous tubules. Sm-actin: smooth-muscle actin. hESC, human embryonic stem cell; hMSC, human mesenchymal stem cell; EB, embryoid body.

In vitro and *in vivo* characterization of the hESC lines derived on hMSCs

AND-1, AND-2 and AND-3 hESC lines have been cultured for over 38 passages so far on hMSCs or in feeder-free conditions using matrigel and MSC-conditioned media (MSC-CM). No differences in *in vitro* and *in vivo* pluripotency and culture homeostasis were observed among these hESC lines maintained on hMSCs and those grown in MSC-CM (data not shown). hESC lines derived on hMSCs retained typical hESC morphology (Fig. 2G, H) and expression of the pluripotency-associated surface markers AP (Fig. 3A), Tra-1-60 (Fig. 3B), Tra-1-81 (Fig. 3C), SSEA-3 (Fig. 3D) and SSEA-4 (Fig. 3E). Immunocytochemistry staining results were confirmed by flow cytometry (Fig. 3; right panels). Similarly, all three hESC lines expressed the transcription factors Oct3/4, Nanog, Rex-1 and Sox-2 (Fig. 4A) and remained karyotypically stable (Fig. 4B). Functionally, these hESC lines successfully differentiated *in vitro* through EB formation (Fig. 5A; left panel) into tissues representing the three germ layers (Fig. 5A): ectoderm (β-III-Tubulin-positive cells), mesoderm (Actin-positive cells) and endoderm (α-fetoprotein-positive cells). The gold-standard pluripotency assay relies on the ability of undifferentiated cells to form teratomas *in vivo* upon injection into immune-deficient mice. The three hESC lines derived and maintained in hMSCs formed teratomas 8–10 weeks after inoculation. These complex and disorganized tumours contained a variety of tissues representing the three germ layers (Fig. 5B), demonstrating the pluripotent features of the hESC lines derived on hMSCs.

Discussion

This study has prospectively examined the use of the ROCK inhibitor Y-27632 and hMSC/HFF feeders on embryo survival and hESC derivation efficiency from a large cohort of poor-quality human embryos which had been cryopreserved for 4–13 years. In addition, two distinct ICM isolation methods (WBC alone versus WBC + LD) were used, based on the embryo quality.

Treatment of cryopreserved embryos with Y-27632 in the culture medium resulted in a 52% increase in embryo survival. Y-27632 has been recently reported to improve the survival of dissociated and cryopreserved hESCs growing in suspension (Watanabe *et al*., 2007; Martin-Ibañez *et al*., 2008; Li *et al*., 2008, 2009). Li *et al*. (2009) have recently proposed through elegant studies a potential mechanism of action for Y-27632. Y-27632 seems to augment survival of hESCs not only by decreasing the level of apoptosis but also through complementary mechanisms, such as increasing cellular adhesion by promoting stronger cell–cell interaction (Li *et al*., 2009). However, to the best of our knowledge, no study has been performed to

determine whether Y-27632 also improves survival of cryopreserved human embryos. The 52% increase in embryo survival we report opens up new avenues for investigators and clinicians, not only in the field of hESC research but also in human assisted reproduction, to explore further potential applications of Y-27632 or analogous compounds in a variety of clinical scenarios. From the clinical standpoint, whether Y-27632 facilitates IVF, embryo development toward blastocyst stage, improves the efficiency of fertilized oocytes achieving the blastocyst stage, or enhances embryo survival upon cryopreservation are still unanswered questions. We envisage that the previously reported data about the role of Y-27632 on hESCs and our data showing the role of Y-27632 in enhancing human embryo survival after cryopreservation will encourage further investigations aimed at optimizing protocols in IVF clinics.

We also demonstrate for the first time that hMSCs may increase the efficiency of hESC derivation from frozen poor-quality embryos as compared with HFFs (10.5% versus 0%). However, further studies are required owing to the number of variables investigated and the limited number of blastocysts/ICM in each arm of our experiment. Three hESC lines (AND-1, AND-2 and AND-3) were successfully derived in hMSCs and have now been karyotypically stable for over 38 passages on hMSCs and have also been successfully transferred to feeder-free culture and maintained in hMSC-CM. The lines have been fully characterized and show typical hESC morphology, euploidy, expression of ESC-associated surface markers and transcription factors, and displayed *in vitro* and *in vivo* multilineage developmental potential. The optimization of hESC derivation methods is advisable because all human embryos are a limited resource and, in many countries, only cryopreserved human embryos (normally of poor-quality) are legally permitted to be used for research (Cortes *et al*., 2007b, 2008). Interestingly, neither the freeze-thaw technique, treatment with Y-27632 nor the ICM isolation method influenced the hESC derivation efficiency. We previously reported with fresh mouse embryos that the efficiency of mESC derivation is superior when WBC is combined with LD (Cortes and Menendez, 2008). However, the LD technology did not offer any improvement over WBC when frozen poor-quality human embryos were used. Laser-assisted derivation of hESCs has been successfully achieved from fresh (better quality) IVF embryos after preimplantation genetic diagnosis (Tanaka *et al*., 2006; Turetsky *et al*., 2008) but caution is recommended when comparing these independent studies as only a few embryos were available. Furthermore, frozen embryos were used in our study whereas fresh embryos were used in the other study. The cellular and molecular mechanisms underlying the role of hMSCs in facilitating embryo development and hESC establishment need to be elucidated. The signalling pathways that regulate embryo development and promote ESC establishment are largely unknown. However, key developmental pathways, including Sonic hedgehog, Notch, Wnt and bone morphogenic proteins, are potential candidates (Bailey *et al*., 2007; Bueno *et al*., 2007). In fact, a number of soluble factors produced by the supporting stroma/MSCs with novel stem cell expansion activities were recently identified as being associated with mesodermal induction (Hutton *et al*., 2007). It should be mentioned that hESC lines have been successfully derived in HFFs using fresh embryos (Inzunza *et al*. 2005; Hovatta, 2006). However, to the best of our knowledge this is the first prospective study comparing two distinct human feeders to improve the derivation of hESC lines from frozen poor-quality embryos.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Acknowledgements

We thank Teresa de la Cueva, Dr Sara Sanbonmatsu, Dr Gema Lucena, Ruth Rubio, Dr Javier García-Castro and Prof. Francisco Nogales for their outstanding technical assistance.

Funding

This work was funded by the Health Department(0028/2006 & 0030/2006 to P.M.) and Innovation Department (P08-CTS-03678 to P.M.) of Andalusian Government, The Spanish Ministry of Health (FIS PI070026 to PM and FIS PI08171 to J.L.G.-P). Research in J.L.G.-P's Laboratory is also supported by the ISCIII-CSJA (EMER07/056) and Marie Curie IRG (FP7-PEOPLE-2007-4-3-IRG). C.B. is supported by the FIS-ISCIII (CP07/00 059) and the Jose Carreras International Foundation against the Leukemia (FIJC-05/EDThomas 2006). V.R.-M. is supported by The Marie Curie (FP7-PEOPLE-IIF-2008). We are indebted to ANACER (Spanish Association of IVF clinics) and Dr Santiago Alvarez (University of Extremadura) for provision of cryopreserved human embryos from couples who voluntary signed an informed consent.

References

Amit M, Margulets V, Segev H, Shakiri K, Laevski I, Coleman R, Itskovitz-Eldor J. Human feeder layers for human embryonic stem cells. *Biol Reprod* 2003;**68**:2150–2156.

Bailey JM, Singh PK, Hollingsworth MA. Cancer metastasis facilitated by developmental pathways: Sonic hedgehog, Notch, and bone morphogenic proteins. *J Cell Biochem* 2007;**102**:829–839.

Bongso A, Fong CY, Ng SC, Ratnam S. Isolation and culture of inner cell mass cells from human blastocysts. *Hum Reprod* 1994;**9**:2110–2117.

Bueno C, Lopes LF, Menendez P. Bone marrow stromal cell-derived Wnt signals as a potential underlying mechanism for cyclin D1 deregulation in multiple myeloma lacking t(11;14)(q13;q32). *Blood Cells Mol Dis* 2007;**39**:366–368.

Bueno C, Montes R, Garcia-Castro J, Greaves M, Menendez P. Human embryonic stem cells: a potential system for modelling infant leukemia harbouring MLL-AF4 fusion gene. *Drug Discov Today: Dis Models* 2008a;**4**:53–60.

Bueno C, Montes R, Martin L, Prat I, Hernandez MC, Orfao A, Menendez P. NG2 antigen is expressed in CD34+ HPCs and plasmacytoid dendritic cell precursors: is NG2 expression in leucemia dependent on the target cell where leukemogenesis is triggered? *Leukemia* 2008b;**22**:1475–1478.

Catalina P, Bueno C, Montes R, Nieto A, Ligero G, Sanchez L, Jara M, Rasillo A, Orfao A, Cigudosa J *et al.* Genetic stability of human embryonic stem cells: a first-step toward the development of potencial hESC-based systems for modeling childhood leukaemia. *Leuk Res* 2008a; DOI: 10.1016/j.leukres.2008.08.028.

Catalina P, Montes R, Ligero G, Sanchez L, de la Cueva T, Bueno C, Leone PE, Menendez P. Human ESC predisposition to karyotipic instability: is a matter of cell culture adaptation or differential vulnerability among hESC lines due to inherent properties? *Mol Cancer* 2008b;**7**:76–82.

Cheng L, Hammond H, Ye Z, Zhan X, Dravid G. Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture. *Stem Cells* 2003;**21**:131–142.

Cobo F, Navarro JM, Herrera I, Vivo A, Porcel D, Hernández C, Jurado M, García-Castro J, Menendez P. Electron microscopy reveals the presence of viruses in MEFs but neither in HFFs or hMSCs used for hESC maintenance: toward an implementation of microbiological quality assurance proANDm in stem cell banks. *Cloning Stem Cells* 2008; **10**:65–74.

Cortes JL, Cobo F, Catalina P, Nieto A, Cabrera C, Montes R, Concha A, Menendez P. Evaluation of the laser technique method to isolate the inner cell mass of murine blastocysts. *Biotechnol Appl Biochem* 2007a; **46**:205–209.

Cortes JL, Antiñolo G, Martinez L, Cobo F, Barnie AH, Zapata A, Menendez P. Spanish stem cell bank interviews examine the interest of couples in donating surplus human IVF embryos for stem cell research. *Cell Stem Cell* 2007b;**1**:17–20.

Cortes JL, Menendez P. Reproductive medicine meets human embryonic stem cell (hESC) research: the need to adjust the regulatory framework to actual expectations and potential detrimental consequences of hESC research. *Fertil Steril* 2008; 10.1016/j.fertnstert.2008.05.041.

Cortes JL, Sánchez L, Catalina P, Cobo F, Bueno C, Martínez-Ramirez A, Barroso A, Cabrera C, Ligero G, Montes R *et al.* Whole-blastocyst culture followed by laser drilling technology enhances the efficiency of inner cell mass isolation and embryonic stem cell derivation from good- and poor-quality mouse embryos: new insights for derivation of human embryonic stem cell lines. *Stem Cells Dev* 2008;**17**: 255–267.

Garcia-Castro J, Trigueros C, Madrenas J, Perez-Simon JA, Rodriguez R, Menendez P. Mesenchymal stem cells and their use as cell replacement therapy and disease modeling tool. *J Cell Mol Med* 2008;**12**:1–14.

Hovatta O. Derivation of human embryonic stem cells: towards clinical quality. *Reprod Fertil Dev* 2006;**16**:823–828.

Hovatta O, Mikkola M, Gertow K, Strömberg AM, Inzunza J, Hreinsson J, Rozell B, Blennow E, Andäng M, Ahrlund-Richter L. A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. *Hum Reprod* 2003; **18**:1404–1409.

Hutton JF, D'Andrea RJ, Lewis ID. Potential for clinical ex vivo expansion of cord blood haemopoietic stem cells using non-haemopoietic factor supplements. *Curr Stem Cell Res Ther* 2007;**2**:229–237.

Inzunza J, Gertow K, Strömberg MA, Matilainen E, Blennow E, Skottman H, Wolbank S, Ahrlund-Richter L, Hovatta O. Derivation of human embryonic stem cell lines in serum replacement medium using postnatal human fibroblasts as feeder cells. *Stem Cells* 2005; **23**:544–549.

Kim HS, Oh SK, Park YB, Anh HJ, Sung KC, Kang MJ, Lee LA, Suh CS, Kim SH, Kim DW *et al.* Methods for derivation of human embryonic stem cells. *Stem Cells* 2005;**23**:1228–1233.

Ledran MH, Krassowska A, Armstrong L, Dimmick I, Renström J, Lang R, Yung S, Santibanez-Coref M, Dzierzak E, Stojkovic M *et al.* Efficient hematopoietic differentiation of human embryonic stem cells on stromal cells derived from hematopoietic niches. *Cell Stem Cell* 2008; **3**:85–98.

Lerou PH, Yabuuchi A, Huo H, Takeuchi A, Shea J, Cimini T, Ince TA, Ginsburg E, Racowsky C, Daley GQ. Human embryonic stem cell derivation from poor-quality embryos. *Nat Biotechnol* 2008; **26**:212–214.

Li X, Meng G, Krawetz R, Liu S, Rancourt DE. The ROCK inhibitor Y-27632 enhances the survival rate of human embryonic stem cells following cryopreservation. *Stem Cells Dev* 2008; **17**:1079–1085.

Li X, Krawetz R, Liu S, Meng G, Rancourt DE. ROCK inhibitor improves survival of cryopreserved serum/feeder-free single human embryonic stem cells. *Hum Reprod* 2009;**24**:580–589.

Martin-Ibañez R, Unger C, Strömberg A, Baker D, Canals JM, Hovatta O. Novel cryopreservation method for dissociated human embryonic stem cells in the presence of a ROCK inhibitor. *Hum Reprod* 2008; **23**:2744–2754.

Menendez P, Wang L, Bhatia M. Genetic manipulation of human embryonic stem cells: a system to study early human development and potential therapeutic applications. *Curr Gene Ther* 2005;**5**: 375–385.

Menendez P, Bueno C, Wang L. Human embryonic stem cells: a journey beyond cell replacement therapies. *Cytotherapy* 2006;**8**:530–541.

Montes R, Ligero G, Sanchez L, Catalina P, de la Cueva T, Nieto A, Melen G, Rubio R, García-Castro J, Bueno C *et al.* Feeder-free maintenance of hESCs in mesenchymal stem cell-conditioned media: distinct requirements for TGF-β and IGF-II. *Cell Res* 2009; doi:10.1038/cr.2009.35.

Narumiya S, Ishizaki T, Uehata M. Use and properties of ROCK-specific inhibitor Y-27632. *Meth Enzymol* 2000;**325**:273–284.

Park SP, Lee KS, Ah Shin H, Cho HY, Chung KS, Kim EY, Lim JH. Establishment of human embryonic stem cell lines from frozen-thawed blastocysts using STO cell feeder layers. *Hum Reprod* 2004;**19**: 676–684.

Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat Biotechnol* 2000;**18**:399–404.

Schäffler A, Büchler C. Concise review: adipose tissue-derived stromal cells-basic and clinical implications for novel cell-based therapies. *Stem Cells* 2007;**25**:818–827.

Solter D, Knowles BB. Immunosurgery of mouse blastocysts. *Proc Natl Acad Sci USA* 1975;**72**:5099–5102.

Tanaka N, Takeuchi T, Neri QV, Sills ES, Palermo GD. Laser-assisted blastocyst dissection and subsequent cultivation of embryonic stem cells in a serum/cell free culture system: applications and preliminary results in a murine model. *J Transl Med* 2006;**4**:20–32.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshal VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science* 1998;**282**:1145–1147.

Turetsky T, Aizenman E, Gil Y, Weinberg N, Shufaro Y, Revel A, Laufer N, Simon A, Abeliovich D, Reubinoff BE. Laser-assisted derivation of human embryonic stem cell lines from IVF embryos after preimplantation genetic diagnosis. *Hum Reprod* 2008;**3**:46–53.

Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease. *Nat Rev Immunol* 2008;**8**:726–736.

Watanabe K, Ueno M, Kamiya D, Nishiyama A, Matsumura M, Wataya T, Takahashi JB, Nishikawa S, Nishikawa S, Muguruma K *et al.* A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nat Biotechnol* 2007;**25**:681–686.

Submitted on February 2, 2009; resubmitted on March 24, 2009; accepted on March 30, 2009