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REPORT

# Derivation and maintenance of human embryonic stem cell line on human adult skin fibroblast feeder cells in serum replacement medium

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**Abstract** Human embryonic stem (hES) cells were originally isolated and maintained on mouse embryonic fibroblast (MEF) feeder layers in the presence of fetal bovine serum (FBS). However, if the hES cells are to be used for therapeutic applications, it is preferable to regulatory authorities that they be derived and cultured in animal-free conditions to prevent mouse antigen contamination that would exacerbate an immune response to foreign proteins, and the potential risk of transmission of retroviral and other zoonotic pathogens to humans. As a step towards this goal, we derived a new hES cell line (MISCES-01) on human adult skin fibroblasts as feeder cells using serum replacement (SR) medium. The MISCES-01 cells have a normal diploid karyotype (46XX), express markers of pluripotency (OCT4, GCTM-2, TRA-1-60, TRA-1-81, SSEA-3, SSEA-4, and alkaline phosphatase) and following in vitro and in vivo differentiation, give rise to derivatives of the three primary germ layers. This cell line can be obtained for research purposes from the Australian Stem Cell Centre (http://www.stemcellcentre.edu.au).

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The unique feature of human embryonic stem (hES) cells is their capacity for self-renewal and pluripotency. Since hES cells can be cultured indefinitely and when differentiated, can potentially give rise to all cell and tissue types of the human body, they offer great promise for regenerative medicine, cell and gene therapies, and disease modeling (Trounson 2006). To fully realize these potentials, hES cells must be derived and maintained in optimal culture conditions that not only preserve the cells' unique characteristics but also make them suitable for clinical applications.

Originally, hES cells were isolated from pre-implantation human embryo in co-culture with mitotically inactivated mouse embryonic fibroblasts (MEF) and in a medium containing fetal bovine serum (FBS) (Thomson et al. 1998; Reubinoff et al. 2000). Both MEF and FBS can represent a source of animal pathogens that can be potentially transmitted to human patients following hES cell-based therapy (Bongso et al. 2008 and references within). The concerns over xeno-contamination led to development of a number of "humanized" culture systems (Mallon et al. 2006 and references within; Lei et al. 2000 and references within). These include derivation of feeder cell lines from adult and fetal human tissues and from differentiated hES cell cultures, substitution of feeders with human- and mouse-derived extracellular matrices, and replacement of FBS with serum replacement (SR). However, the ultimate goal is to develop a methodology that will allow generation of clinical-grade hES cells in defined conditions totally free of animal products. As a step towards this goal, we undertook experiments to derive hES cells on commercially available human adult skin fibroblast feeders and in a medium containing SR. Here, we report the results of this isolation.

In Australia, research involving human stem cells is regulated by the Australian Commonwealth Research Involving Human Embryos Act 2002. The Monash University Standing Committee on Ethics in Research Involving Humans (SCERH) and the National Health and Medical Research Council for Embryo Research licensing committee approved the isolation and characterization of hES cells. Embryos surplus to in vitro fertilization (IVF) treatment were donated (without financial inducements) by couples following an informed consent. The consent did not impose any restrictions on the subsequent use of the hES cell lines.

All chemicals were obtained from Gibco, BRL Life Technologies, Melbourne, Victoria, Australia, unless otherwise indicated.

The embryos were produced following routine procedures for clinical IVF by the Monash IVF Pty Ltd clinic. Two to 9 d post fertilization, two- to ten-cell stage embryos were frozen and kept in cryostorage for between 1 and 9 yr. The donated embryos were thawed using the Sydney IVF thawing kit (K-SITS-5000, Cook Australia Pty Ltd) and placed onto four-well dishes (Nunc, Roskilde, Denmark) in 50 μL drops of either 'single step' (Global Medium GMGB-050, LifeGlobal, the ART Media Company, Canada) or 'sequential' (Sydney IVF Cleavage K-SICM-50 and Blastocyst Medium K-SIBM-50, Cook Australia Pty Ltd) embryo culture medium. The drops were covered with 200 μL of oil (Sydney IVF Culture Oil K-SICO-200, Cook Australia Pty Ltd.). The embryos were incubated at 37°C in an atmosphere of 5% $O_2$; 5% $CO_2$, 90% $N_2$ with 95% relative humidity.

Human skin fibroblasts (CCD919SK CRL-1826, ATCC) were used as feeder cells. The fibroblasts were maintained in alpha minimum essential medium (α-MEM) supplemented with 10% FBS, 50 IU $mL^{-1}$ penicillin and 50 μg $mL^{-1}$ streptomycin. To prepare the feeder layers, $7.5\times10^4$ cells per $cm^2$, mitotically inactivated with mitomycin (10 μg $mL^{-1}$; Sigma), were plated onto organ culture dishes (BD Biosciences).

Derivation and culture of hES cells was carried out in Knockout-Dulbecco's modified Eagle's medium (KO-DMEM), supplemented with 20% Knockout-Serum Replacement (KOSR), 1% nonessential amino acids (NEAA), 1% insulin-transferrin-selenium (ITS), 2 mM glutamine, 0.1 mM 2-mercaptoethanol, 50 IU $mL^{-1}$ penicillin, 50 μg $mL^{-1}$ streptomycin, and 4 ng $mL^{-1}$ recombinant human basic fibroblast growth factor (hbFGF; R&D Systems) (Amit et al. 2000). The cells were incubated at 37°C in an atmosphere of 5% $O_2$; 5% $CO_2$, 90% $N_2$ with 95% relative humidity.

In the first series of experiments, we attempted to derive hES cells from morula stage embryos. Out of 12 embryos, eight survived thawing and were placed in a 'single step' embryo culture medium and cultured for 30–48 h. Five embryos developed to the compacted morula stage, two embryos developed to the 16-cell stage without compaction, and 1 embryo showed no further cleavage. The 16-cell and compacted morula stage embryos were cultured under or above the human fibroblast feeder layer (Vajta et al. 2000; Strelchenko et al. 2004). Two embryos cultured under feeder layer showed further development and were passaged onto new feeder cells after 10 d, however, colonies failed to grow following two passages.

Since derivation of hES cells from the morula stage embryos was unsuccessful, in the second series of experiments, we cultured thawed embryos to the blastocyst stage. Out of 34 embryos, 33 survived thawing and were placed in a 'sequential' embryo culture medium and incubated for 48–96 h. The majority of embryos (20/33) have arrested at various stages of development while 13 embryos reached blastocyst stage, all judged to be of poor quality. Inner cell mass (ICM) was mechanically dissected from two blastocysts following removal of zona pellucida with 0.5% protease (Sigma). However, both ICM failed to attach to feeders.

Due to poor quality, the remaining embryos were plated as whole on the feeder layers. Half of the medium was changed initially every day starting 48 h after plating and then a complete medium change was performed after 96 h of culture. The first passage of ICM outgrowths was performed 7–8 d after plating by cutting outgrowths with an ultra-sharp splitting blade into two to five fragments and transferring the fragments to a fresh feeder layer. Out of 11 embryos plated, one gave rise to an established hES cell line (MISCES-01), while the remaining putative hES cells failed to grow after two to four passages. The MISCES-01 cell line has been maintained by mechanical splitting of morphologically undifferentiated cell fragments every 7 d. The MISCES-01 cells have a high nucleus to cytoplasm ratio; the colonies are flat and thin and grow according to the pattern of the human feeders (Fig. 1*A*). The MISCES-01 cell line was cryopreserved by vitrification in pulled open straws (Reubinoff et al. 2001) at passages 4, 7, 8, 9, 11, 20, 21 and successfully thawed at passages 4 and 8.

The MISCES-01 cell line was characterized by indirect immunocytochemistry. The cells were fixed with 100% methanol and incubated with primary antibodies (SSEA-1, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 [Chemicon]; OCT-4 [Santa Cruz Biotechnology Inc.]; GCTM2 [gift from M. Pera, Monash University, Australia]) followed by the incubation with FITC- or TRITC-conjugated secondary antibody (Daco Corporation and Santa Cruz Biotechnology Inc., respectively). The nuclei of the cells were counterstained with 4′-6-diamidino-2-phenylindole (DAPI; Sigma). The activity of alkaline phosphatase was determined using alkaline phosphatase detection kit according to manufacturer's instructions (Roche Applied Science). The MISCES-

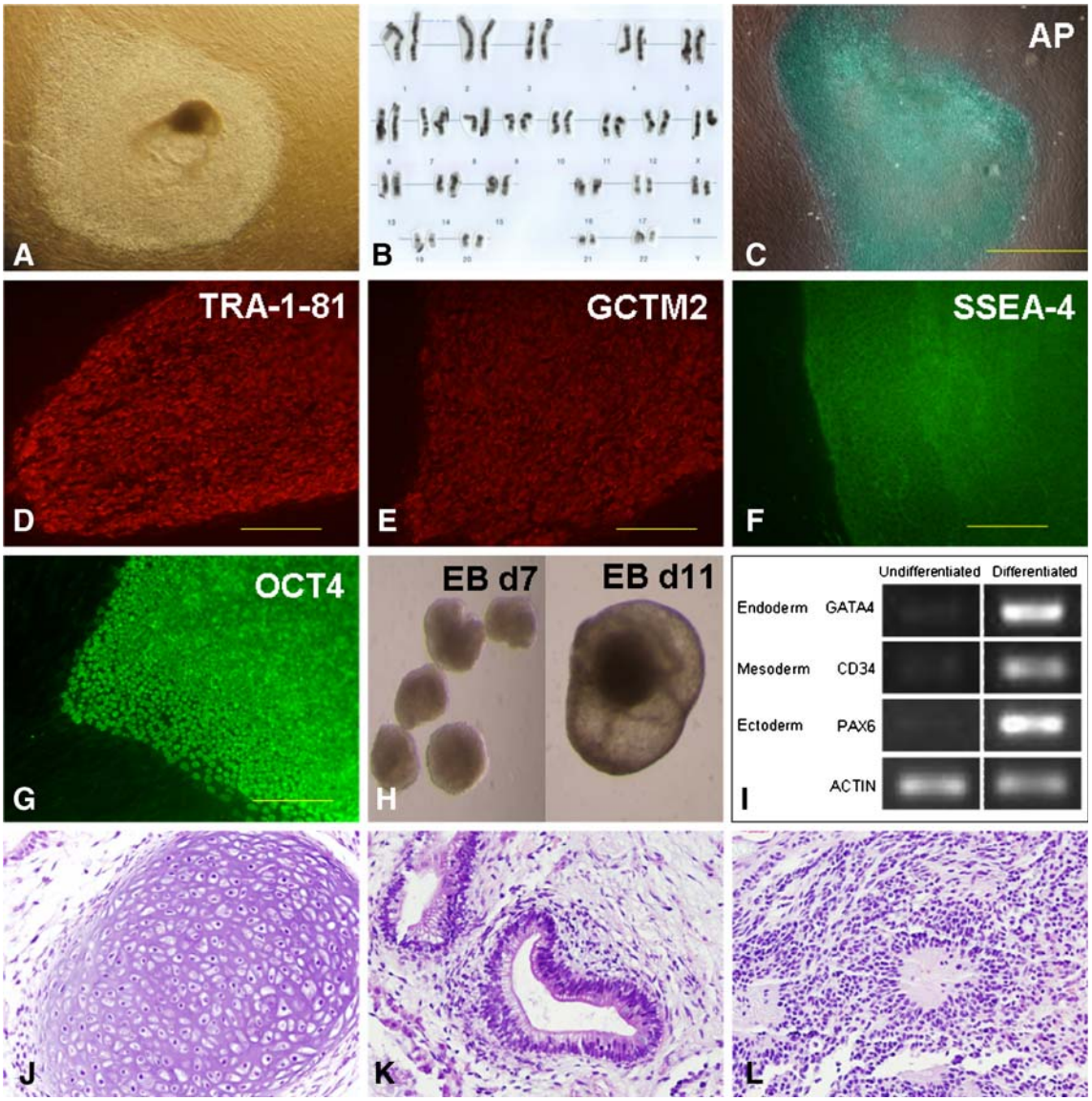


**Figure 1.** Morphology and characterization of hES cell line MISCES-01. (*A*) Phase contrast image showing morphology of undifferentiated colony. Magnification 10x. (*B*) Giemsa band karyogram showing normal female karyotype. (*C–G*) Microscopic images showing undifferentiated colony positive for (*C*) alkaline phosphatase, (*D*) TRA-1-81, (*E*) GCTM2, (*F*) SSEA-4, (*G*) Oct4. (*H–I*) Embryoid body-mediated differentiation of MISCES-01 cells. (*H*) Morphology of embryoid bodies (*EB*) on day 7 (*d7*) and day 11 (*d11*) of culture. Magnification ×10. (*I*) RT-PCR analysis of in vitro EB-differentiated MISCES-01 cells showing transcript expression of GATA4 (endoderm), CD34 (mesoderm), and PAX6 (ectoderm). ACTIN is shown as a positive amplification and loading control. (*J–L*) Histology of teratomas formed from cells grafted in testis showing (*J*) cartilage [mesoderm], (*K*) secretory epithelium [endoderm], (*L*) neuroectoderm [ectoderm]. *Scale bar* (*C*) 500 μm; (*D–G*) 200 μm.

01 cells expressed pluripotent markers OCT4, SSEA-3 (not shown), SSEA-4, TRA-1-60 (not shown), TRA-1-81, GCTM2, alkaline phosphatase (Fig. 1*C–G*) and tested negative for the expression SSEA-1 (not shown).

To demonstrate the potential of MISCES-01 cells to differentiate in vitro, the pieces of hES cell colonies were grown in suspension in the absence of feeders in a culture medium without hbFGF. Under these conditions, by day 7 of culture, the MISCES-01 cells formed simple embryoid bodies (EBs) that expanded and became cystic by day 11 (Fig. 1*H*). Total RNA was extracted from day 11 EBs using PicoPure RNA isolation kit (Arcturus) followed by complementary DNA (cDNA) synthesis (SuperScript III First Strand Synthesis System, InVitrogen) according to manufacturer's instructions. The expression of marker genes characteristic of development into three germ layers was analyzed by RT-PCR (GATA4, sense: CTAGACCGTGGG TTTTGCAT, antisense: TGGGTTAAGTGCCCCTGTAG; CD34, sense: GCCATTCAGCAAGACAACAC, antisense: AAGGGTTGGGCGTAAGAGT; PAX6, sense: AACAGA

CACAGCCC TCACAAACA, antisense: CGGGACT TGA AC TGGAACTGAC). Beta actin, a housekeeping gene (sense: CAGAGCCTCGCCTTTGC CG, antisense: CTCGCGG TG GCCTTGGG) was used as a positive control. Endoderm, mesoderm, and ectoderm specific markers were detected.

The in vivo pluripotency of the MISCES-01 cell line was tested by inducing teratoma formation in severe combined immunodeficiency (SCID; NOD.CB17-prkdcscid/Jasmu) male mice. All animal experiments were approved by the Monash University Animal Ethics Committee in accordance with the Australian Code of Practice for the care and use of animals for scientific purposes. Approximately $5\times10^4$ undifferentiated cells were injected into the testis of 5-wk old SCID mice. Resulting teratomas were removed after 6–8 wk, fixed in HistoChoice (Amresco) and embedded in paraffin. Histological examination performed on hematoxylin-and-eosin-stained sections revealed that teratomas consisted of derivatives of all three primary germ line lineages (Fig. 1*J–L*).

The karyotyping of the MISCES-01 was performed at passages 6 and 19 using a standard G-banding technique. The analysis of 20 metaphase spreads has revealed a normal female karyotype (46XX; Fig. 1*B*).

The MISCES-01 cell line has been tested and declared free of mycoplasma and common sexually transmitted pathogens (herpes simplex virus types 1 and 2, varicella zoster virus [herpes zoster], human immunodeficiency virus [HIV], hepatitis B and C).

In summary, we report successful derivation of a new hES cell line on commercially available human adult skin fibroblasts in a medium containing serum replacement. The MISCES-01 cells express markers typical of undifferentiated hES cells, have a normal diploid karyotype and retain ability to differentiate both, in vitro and in vivo into tissues representative of endoderm, mesoderm, and ectoderm (Fig. 1). The cells have been in continuous culture for 38 passages, and have been frozen and successfully cultured following thawing. The MISCES-01 cell line is available from the ASCC (http://www.stemcellcentre.edu.au) to researchers subject to a standard Material Transfer Agreement.

The efficiency of hES cell line isolation depends on a number of factors, in particular the quality and the developmental stage of embryos and the procedures used in the initial stages of embryo culture. Cleavage-stage embryos (two- to ten-cell stage following 2 to 9 d post fertilization) used in this study were assessed to be of poor quality following thawing and culture. This could explain our failure to derive hES cells from morula stage embryos and a low number of embryos developing to a blastocyst stage in a 'sequential' medium.

Serum replacement and various human feeders have been used for the maintenance of established hES cells (Lei et al. 2000 and references within; Mallon et al. 2006 and references within). However, there are limited reports of derivation of hES cells using these conditions (Genbacev et al. 2005; Inzunza et al. 2005; Lee et al. 2005). Some success of derivation and propagation of hES cells in a medium containing human serum has also been reported, but hES cells grown under these conditions are prone to undergo extensive differentiation (Rajala et al. 2007 and references within). Until a defined human serum replacement is developed, a semi-defined FBS-derived KOSR is a protein supplement of choice for the isolation and culture of hES cells. At the time of derivation of the MISCES-01 cell line reported here, no clinical-grade human fibroblasts were commercially available and due to a significant work effort and cost required, we have not attempted to establish such a line. Therefore, we derived a new hES cell line (MISCES-01) on commercially available human adult skin fibroblasts in a medium containing SR. Although these conditions are not entirely free of animal-derived components since fibroblasts have been cultured in a medium containing FBS and SR contains AlbuMAX, a lipid-rich albumin fraction of bovine serum and other proteins (Price et al. 1998), they, nevertheless, provide an important step towards isolation of clinical-grade hES cells.

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