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REPORT

Generation of Sheffield (Shef) human embryonic stem cell lines using a microdrop culture system

Behrouz Aflatoonian · Ludmila Ruban · Shamsul Shamsuddin · Duncan Baker · Peter Andrews · Harry Moore

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Abstract The conventional method for the derivation of human embryonic stem cells (hESCs) involves inner cell mass (ICM) co-culture with a feeder layer of inactivated mouse or human embryonic fibroblasts in an *in vitro* fertilisation culture dish. Growth factors potentially involved in primary derivation of hESCs may be lost or diluted in such a system. We established a microdrop method which maintained feeder cells and efficiently generated hESCs. Embryos were donated for stem cell research after fully informed patient consent. A feeder cell layer was made by incubating inactivated mouse embryonic fibroblasts (MEFs) feeder cells in a 50 μl drop of medium (DMEM/10% foetal calf serum) under mineral oil in a small tissue culture dish. MEFs formed a confluent layer and medium was replaced with human embryonic stem medium supplemented with 10% Plasmanate (Bayer) and incubated overnight. Cryopreserved embryos were thawed and cultured until the blastocyst stage and the zona pellucida removed with pronase (2 mg/ml; Calbiochem). A zona-free intact blastocyst was placed in the feeder microdrop and monitored for ES derivation with medium changed every 2-3 d. Proliferating hESCs were passaged into other feeder drops and standard feeder preparation by manual dissection until a stable cell line was established. Six hESC lines (Shef 3-8) were derived. From a total of 46 blastocysts (early to expanded), five hESC lines were generated (Shef 3-7). Shef 3-6 were generated on MEFs from 25 blastocysts. Shef7 was generated on human foetal gonadal embryonic fibroblasts from a further 21 blastocysts. From our experience, microdrop technique is more efficient than conventional method for derivation of hESCs and it is much easier to monitor early hESC derivation. The microdrop method lends itself to good manufacturing practice derivation of hESCs.

Keywords Blastocyst · Derivation · Differentiation · Inner cell mass · Microdrop system · Open system · Human embryonic stem cells (hESCs)

B. Aflatoonian (✉) · L. Ruban · S. Shamsuddin · D. Baker · P. Andrews · H. Moore
Centre for Stem Cell Biology, Sheffield University, Sheffield S10 2TN, UK
e-mail: b.aflatoonian@gmail.com

Present Address:
B. Aflatoonian
IVF Unit, Madar Hospital, Yazd, Iran

Introduction

Human embryonic stem cells (hESCs) provide a source of pluripotent cells for a wide variety of investigations on human embryogenesis and development, aspects of cell signalling, cell cycle, apoptosis, as well as for cell therapy, drug discovery and toxicology screening (Jensen et al. 2009).

The first successful derivation of hESC lines was reported by Thomson and co-workers (Thomson et al. 1998) where isolated inner cell mass (ICMs) were plated onto mitotically inactivated mouse embryonic feeders (MEFs) in culture medium with basic fibroblast growth factor and foetal calf serum. Leukaemia inhibiting factor was added to the original culture conditions but this growth factor was shown not to be required for hESCs in contrast to mESCs (Smith et al. 1988). The basics of this derivation method have become a common approach in the majority of laboratories for derivation of hESCs (Moon et al. 2006).

Reports have shown derivation of hESCs from normal, chromosomally aneuploid and mutant embryos, which are accessible after *in vitro* fertilisation (IVF) for infertility or pre-implantation genetic diagnosis (Trounson 2005). These hESC lines have a promising potential for use as a model for the study of human cellular and molecular development.

Success in the derivation of hESC lines depends in part on the quality of the human embryos used (usually blastocysts from day 5-8) although cell lines have been generated from morphologically poor embryos. It is important to have good knowledge of the *in vitro* culture conditions for embryo development that are employed to generate good quality blastocysts (Bongso 2006). Several reports have shown that co-culture with different cell lines improves *in vitro* embryo development in different species. Usually, it has been performed in an open culture without a mineral oil cover or in comparatively large volumes of medium (e.g. 0.5 ml) under oil (Sherbahn et al. 1996). Both co-culture and microdrop culture systems significantly improved blastocyst development. The combination of the two techniques (microdrop and co-culture) demonstrated the highest blastocyst formation and hatching blastocyst formation rates, as well as the highest mean cell numbers in hatching blastocysts. Co-culture in a microdrop is a superior system for mouse embryo culture (Sherbahn et al. 1996). More recently, microdrop procedures have used to generate hESC lines from blastomeres (Chung et al. 2008)

Here, we describe the generation of Sheffield (Shef) hESC lines. With the exception of Shef 1 and 2, these were produced using a microdrop method.

Methods and Results

Consent and donation of embryos. Embryo research in the UK is carried out under the licence from the Human Fertilization and Embryology Authority following independent ethical review. Patients donate embryos after fully informed consent. For research purposes, all embryos were coded for patient anonymisation.

Embryo culture and derivation of hESCs. Cryopreserved embryos were thawed using an embryo thawing pack (MediCult, UK) and cultured in a 50 µl microdrop system until the blastocyst stage with G series medium (version III; Vitrolife) +5% human serum albumin (HSA; Vitrolife).

Generation of Shef 1 and 2. The first two cell lines of the Sheffield series were produced by a modified conventional method (Thomson et al. 1998). MEFs were derived and cultured as described elsewhere (Draper et al. 2004). Cells were inactivated by γ-irradiation (35 greys) for 10 min. Adherent cells were recovered from the flask, washed by centrifugation, and resuspended in 10 ml of DMEM/10% foetal calf serum (FCS) and distributed in the gelatin-coated central well of IVF Petri dishes (3×10^5/ml) for incubation at 37 C at 5% CO_2 in air.

ICMs of blastocysts were isolated by immunosurgery. The zona pellucida of embryos was removed by incubation with 2 mg/ml pronase at room temperature (Calbiochem; prepared previously in 30 µl microdrops of culture medium covered by mineral oil) in embryo culture medium. Zona-free blastocysts were washed from pronase by aspiration and transferred through five microdrops of human embryonic stem (HES) medium (Thomson et al. 1998) covered by mineral oil followed by incubation in a 30 µl microdrop of anti-SSEA1 antibody (specific for trophectoderm) as hybridoma supernatant (dilution 1:5) for 30 min and washed by aspiration through a further five microdrops of HES medium covered by mineral oil. Trophoblast cell lysis was carried out with baby rabbit complement (Harlan Sera-lab, UK)) in a microdrop (dilution1:4 in HES medium). The embryo was recovered and gently aspirated in HES medium to remove lysed trophoblast cells and free the ICM which was cultured on MEFs feeders with HES medium with 10%FCS for Shef1, and 20% knock out serum replacement (KOSR; Invitrogen) for Shef2.

Generation of Shef 3-8. To improve the efficiency of derivation intact zona-free blastocysts were used in a microdrop (Fig. 1). MEFs were prepared as above and then seeded into 50 µl microdrops of medium at an initial density of 3×10^5/ml and incubated at 37 C at 5% CO_2 in air overnight. The following day, the medium was replaced with HES medium. The zona pellucida of embryos were removed as above and zona-free blastocysts and plated into the microdrops which were covered with embryology mineral oil (Ovoil; Vitrolife) and incubated at 37 C in 5% CO_2 in air. Small modifications to the technique for different cell lines are shown in Table 1.

Establishment of Shef lines. To consolidate a new cell line after obtaining a preliminary hESC colony, the cells need to be established for several passages. Furthermore, cells must be cryopreserved, karyotyped, and initially characterised using immunostaining with specific markers. The pluripotency of new cell lines can be assessed *in vitro* by making embryoid bodies and assessing their spontaneous differentiation to all three germ layers.

Shef lines were all cultured on inactivated MEFs except for the preliminary colony of Shef 7 which was grown on human foetal gonadal fibroblasts (HFGFs) feeder layer. Procedures of culturing and maintenance of hESC lines were as described in elsewhere (Draper et al. 2004: Avery et al. 2008; Furue et al. 2008). HESCs

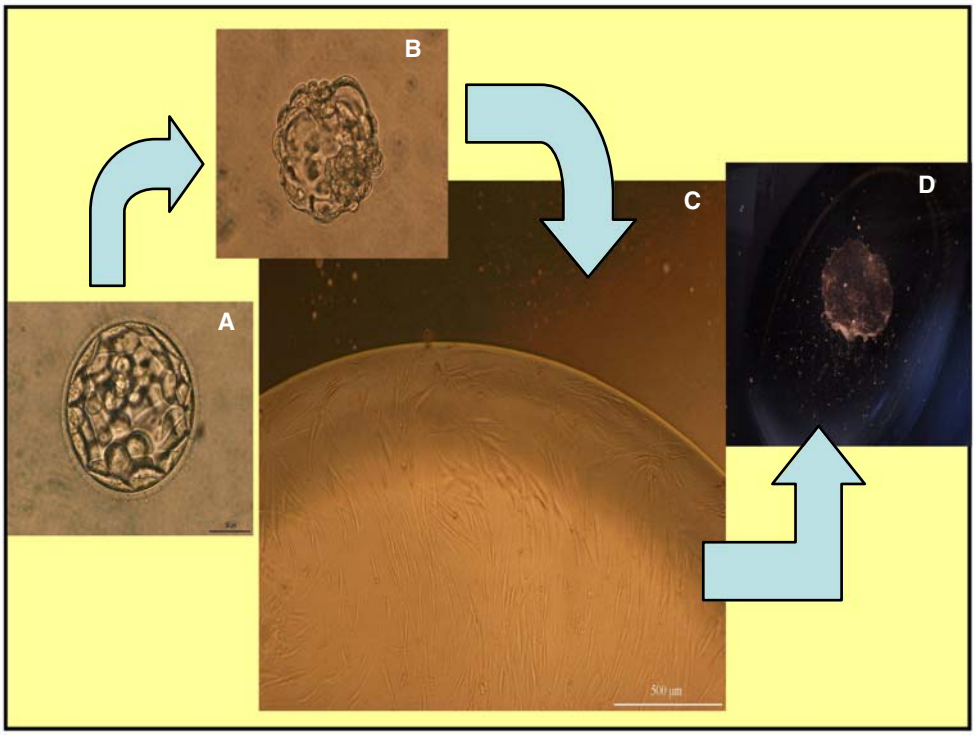

Figure 1. Micrographs of microdrop method for derivation of Shef lines. (*A*) Day 6 intact blastocyst was treated with pronase to remove (*B*) the zona pellucida and transferred to (*C*) 50 µl microdrop of feeders. (*D*) HESCs may develop after 5-10 d and can be identified under dark field microscopy.

required daily feeding with fresh medium to maintain their undifferentiated state. This was achieved by replacing half the medium. Areas of spontaneous differentiation were removed from the cultures by picking with a sterile pipette. After 5-10 passages (depending on the quality of the cell line in proliferation and differentiation properties), collagenase digestion was applied to detach the undifferentiated hESC colonies. This involved treatment with collagenase type IV for 10-12 min at 37 C at 5% CO_2 in air. After enzymatic treatment, the medium was replaced with HES medium and colonies were detached gently by plastic pipette (or glass beads in higher passages) and washed by centrifugation in HES medium and transferred on new feeders with HES medium. The initial outgrowths of hESCs were detected between 3 and 10 d after initial embryo manipulation. Examples of initial clones are shown in Fig. 2.

Cryopreservation and thawing of Shef lines. Cell lines differed in the initial rate of proliferation and differentiation, but usually showed consistent culture by p6-10 at which stage they were cryopreserved. Master stocks were cryopreserved by the open pulled-straw method (Reubinoff et al. 2001). Briefly, embryo straws (Cryobiosystems, IMV Technologies, Paris, France) were gently heated and pulled out to half their diameter. Small clumps of hESCs were recovered by scraping and placed in a small drop of medium (with HEPES buffer). The cells were transferred to vitrification medium containing ethylene glycol and DMSO first at 10% and then at 20%. The cells were drawn into the prepared straws by capillary action and plunged into liquid nitrogen for vitrification and transferred to long cryovials which were sealed with a cap and placed in a liquid nitrogen storage vessel. For thawing, a straw was taken from a vial, allowed to warm in air for 10 s and then the

Table 1. Modifications to derivation technique for Shef lines

Cell line	Fresh embryo	Frozen embryo	Feeder	ICM isolation	Intact blastocyst	Serum supplement
Shef 1	+	−	MEFs (open)	+	−	FCS
Shef 2	+	−	MEFs (open)	+	−	KOSR
Shef 3	−	+	MEFs (microdrop)	−	+	KOSR + plasmanate
Shef 4	−	+	MEFs (microdrop)	−	+	KOSR + plasmanate
Shef 5	−	+	MEFs (microdrop)	−	+	KOSR + plasmanate
Shef 6	+	−	MEFs (microdrop)	−	+	KOSR + plasmanate
Shef 7	+	−	HFGFs (microdrop)	−	+	KOSR + plasmanate
Shef8	+	−	MEFs (microdrop)	−	+	KOSR

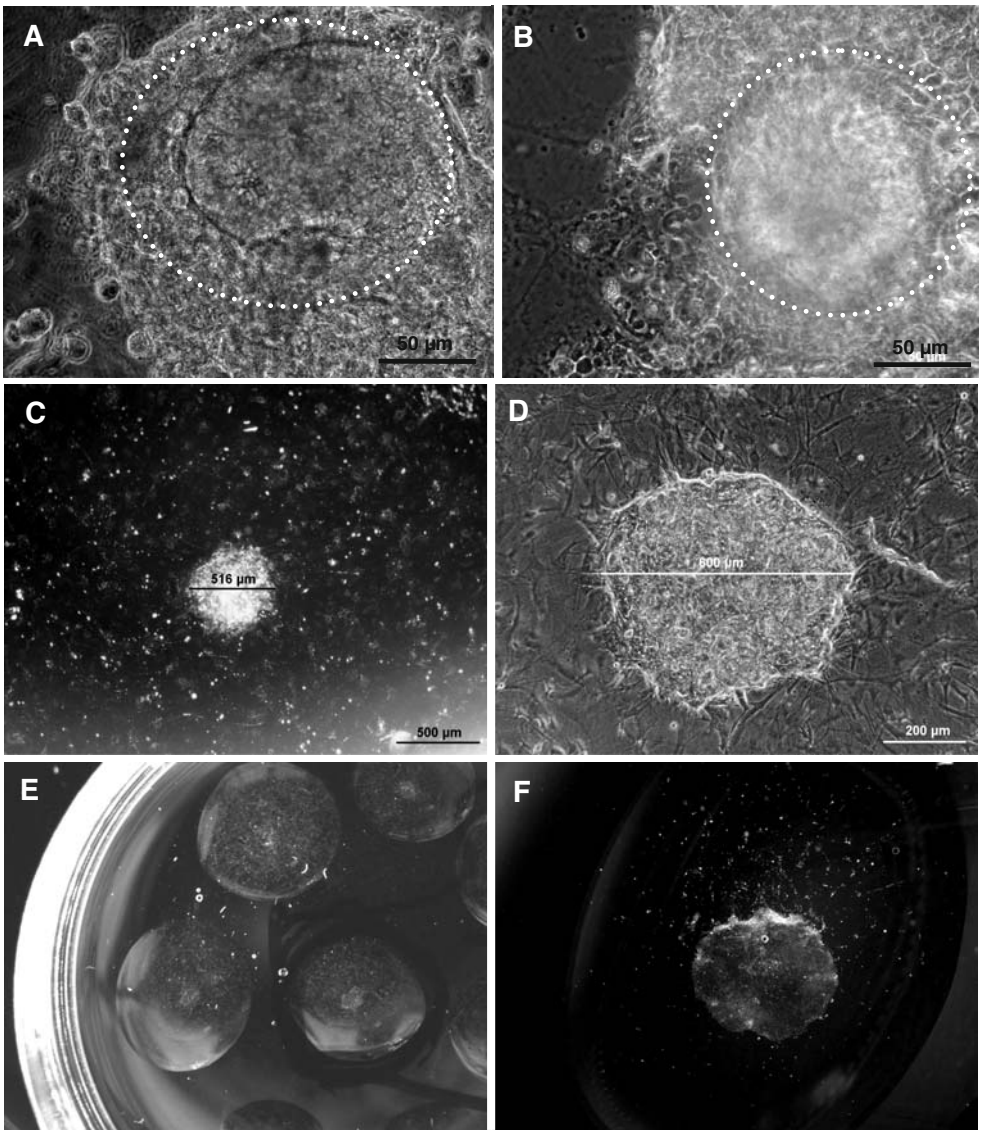

Figure 2. Examples of initial colonies of Shef lines in microdrop. Shef3 (*A*) and Shef6 (*B*) were derived from preliminary colonies with similar morphology. Dense parts within each colony (*A* and *B*, *white dotted circles*) from which after passaging hESCs became established. The size of the first colony of Shef4 (*C*) and Shef 5 (*D*) after 5 d was 516 µm and 600 diameters, respectively. View of hESC colonies in microdrop of feeders in low (*E*) and high (*F*) magnifications.

open end place in a drop of HES medium and agitated so that the clumps of cells went into the drop. The cells were washed by transfer through five times of drops and then placed into medium at 37 C at 5% CO_2 in air.

Characterisation of Shef lines. Cell surface antigen expression confirmed hESC characteristics (Table 2). Shef 1-3 were examined in the International Stem Cell Initiative project to compare cell lines and subjected to extensive profiling (Adewumi et al. 2007). Karyotype was established by G-banding technique (Table 2). The pluripotency of all cell lines has been established *in vitro* using various monolayer and EB differentiation protocols with expression of various genes for major lineages confirmed in all cell lines as shown in Table 3. Teratoma formation in mouse has not been undertaken. All Shef cell lines are deposited in the UK Stem Cell Bank (www.ukstemcellbank.org.uk) and can be readily accessed for research.

Discussion

Following generation of hESC lines by the conventional method (Shef 1 and 2), we have used a microdrop method which gives good efficiency with cryopreserved thawed embryos. The process of derivation is related to the source of hESCs and these can be pre-implantation embryos from morula stage (Strelchenko et al. 2004) up until blastocyst stages after 8 d (Stojkovic et al. 2004). Derivation of a new hESC line is a different process to the culture and maintenance of an established hESC line as the transformation of ICM to hESCs must be considered a system which is a combination between embryo and stem cell culture. A microdrop system was used as had been first applied for animal systems. First and colleagues (First et al. 1994) showed that using microdrops improved bovine embryonic cell culture. In addition, growth factors potentially involved in primary derivation of hESCs may be lost

Table 2. Summary of marker and karyotype characterisation of Shef lines

Cell line	SSEA1	SSEA3	SSEA4	TRA-1-60	TRA-1-81	TRA-2-49	TRA-2-54	OCT4	NANOG	SOX2	Karyotype
Shef 1	−	+	+	+	+	+	+	+	+	+	46, XY
Shef 2	−	+	+	+	+	+	+	+	+	+	46, XX
Shef 3	−	+	+	+	+	+	+	+	+	+	46, XY
Shef 4	−	+	+	+	+	+	+	+	+	+	46, XY
Shef 5	−	+	+	+	+	+	+	+	+	+	46, XX
Shef 6	−	+	+	+	+	+	+	+	+	+	46, XX
Shef 7	−	+	+	+	+	+	+	+	+	+	46, XX
Shef 8	−	+	+	+	+	+	+	+	+	+	46, XX

or diluted in an open system, as it has been shown in comparison of mouse embryo development in open and microdrop co-culture system (Sherbahn et al. 1996).

In some recent reports, it has been shown the use of laser dissection for isolation of ICM in the mouse (Cortés et al. 2007; Cortes et al. 2008) and human (Tanaka et al. 2006; Turetsky et al. 2008) may be useful. Partial or whole blastocyst culture (in an open system) has also been used for the derivation of hESCs. In the present study, the first two hESC lines (Shef1 and Shef2) were derived using the conventional method of immunosurgery. However, to improve the rate of derivation, a microdrop system was devised without isolation of ICM and without considering the stage of embryo development (embryos from morula stage and blastocysts from early cavity (Shef 6) till expanded (Shef 7) and hatched (no cell line) blastocysts. Because the whole zona-free blastocyst was used, the risk of trophectodermal overgrowth, which in some cases causes the differentiation of the preliminary outgrowths, may have increased. However, this was compensated for by the minimal manipulation of the embryo to maintain viability.

The research grade Shef lines described were produced from December 2003 to 2007. The initial rate of cell lines per embryo (NB not blastocyst) was 4.8% but improved threefold to ~16% with the microdrop technique using MEFs. Overall, the results are in keeping with the finding of other studies although optimal timing and use of laser dissection to may improve rates further (Chen et al. 2009). The rate drops to about 3.5% of embryos for production of stable cell lines of normal karyotype after 10-15 passages.

While hESCs are usually derived from the ICM, several reports describe derivation from single blastomeres (and aggregates) of cleavage or morula stage embryos (Strelchenko et al. 2004; Klimanskaya et al. 2006: Klimanskaya et al. 2007; Chung et al. 2008) involving co-culture with feeder cells or with hESCs. Recently, Wakayama and co-workers (Wakayama et al. 2007) described an efficient micro-method of establishing mESC lines from single blastomeres of two-cell embryos (establishment rate, 50-69%), plated on feeders of MEFs on 96-well plates. The use of early cleavage stage human embryos for derivation might increase efficiency considerably as only about a third of all human embryos in culture reach late blastocyst stage. In contrast, mESCs have been derived equally efficiently from late blastocysts with epiblast development and hESCs have been produced from late stage blastocysts (Stojkovic et al. 2004). Therefore, it has been wondered that some hESC characteristics can be attributed to an epiblast epithelial-like origin. Recently, rodent epiblast stem cell lines have been produced from rat and mouse embryos with characteristics more similar to hESCs than conventional mESCs (Brons et al. 2007; Tesar et al. 2007). Thus, it is imperative to understand the exact route of derivation and determine for example whether extracted human blastomeres develop to ICM/epiblast cells and then transform to hESCs or whether blastomeres may directly alter to a potential hESC phenotype.

We are now applying the microdrop system from derivation of hESCs to clinical good manufacturing practice standard using human foreskin feeders.

Table 3. Summary table of gene expression (RT-PCR) after differentiation in embryoid body or monolayer culture

Positive expression has been confirmed for various genes in all Shef cell lines

Endoderm	Mesoderm	Ectoderm	Trophoblast Cells	Germ cells
Ck-18	Cardiac-actin	Neurod1	Hcg-beta	Dazl
Glut-1	Brachyury	Nestin	Cdx2	Vasa
Pax4	Wt1	Pax6	Eomesodermin	Prm1
Insulin	Myod1		Ck7	Tnp2
Pdx1	Desmin			Gdf9
				Zp1

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