Article

Preimplantation genetic diagnosis as a novel source of embryos for stem cell research

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Abstract

The generation of human embryonic stem (hES) cells has captured the public and professional imagination, largely due their potential as a means of overcoming many debilitating and degenerative diseases by cell replacement therapy. Despite this potential, few well-characterized hES cell lines have been derived. Indeed, in the UK, despite several centres having been active in this area for more than 2 years, there are as yet no published reports of human embryonic stem cells having been generated. Part of the reason for this lack of progress may relate to the quality of embryos available for research. Embryos surplus to therapeutic requirements following routine assisted reproduction treatment are often of poor quality and a large proportion may be aneuploid. This study reports a new approach to hES cell derivation. Embryos surplus to therapeutic requirements following preimplantation genetic diagnosis were used. Although unsuitable for embryo transfer due to the high risk of genetic disease, these embryos are from fertile couples and thus may be of better quality than fresh embryos surplus to assisted reproduction treatment cycles. Embryos donated after cryopreservation were also used, and putative hES lines were derived from both sources of embryos. The cell lines described here are thought to be the first reported hES cell lines to have been derived in the UK.

Keywords: blastocyst, human embryonic stem cell lines, PGD

Introduction

The development of human embryonic stem (hES) cell technology has been heralded as the dawn of a new era in cell transplantation therapy, drug discovery and genomics (Keller and Snodgrass, 1999; Odorico et al., 2001). However, to date only a handful of laboratories worldwide have been able to derive these cells successfully. In part, this may be due to the poor quality of embryos available for this work. During assisted reproduction treatment, several embryos are usually generated and cultured for 2–5 days prior to embryo replacement so that those of best quality can be discriminated from those of poorer quality, which are often subject to developmental arrest (Bolton et al., 1989). This approach gives the patient the best chance of pregnancy. Once replacement is complete, remaining embryos of good or fair quality are usually cryopreserved for future therapeutic use (Mandelbaum et al., 1987). Embryos that have not been replaced or cryopreserved are usually discarded, or may be donated to research, at the patient’s discretion. Embryos in this category will have been classified by the clinical embryologist as unlikely to be progressive or able to survive the cryopreservation process. Such embryos are usually of poor quality and often are aneuploid (Munné et al., 1994). Although they may still be useful in many research projects, for hES derivation a progressive, good quality embryo capable of reaching the blastocyst stage and with a discernible inner cell mass is preferable. Embryos reaching this stage after a normal assisted reproduction treatment cycle should still be of use to the patient in their own treatment, even if frozen, making it increasingly difficult to justify the use of such embryos for research. It was decided to address this problem by utilizing...
embryos surplus to therapeutic requirements following pre-implantation genetic diagnosis (PGD) as an ethical alternative for the procurement of research material for stem cell derivation.

PGD is a procedure which allows embryos to be tested for the presence of a specific genetic defect prior to implantation, and was developed as an alternative to prenatal diagnosis to reduce the transmission of severe genetic disease in fertile couples carrying a genetic disorder (Braude et al., 2002). In PGD, a number of embryos are generated by routine assisted reproduction treatment and cellular material is then taken as a biopsy from oocytes or early human embryos that have been cultured in vitro (De Vos and Van Steirteghem, 2001). The biopsy is tested for the presence of a specific genetic abnormality and on the basis of the test results, a maximum of two unaffected or low risk embryos are selected for transfer to the uterus. During this process, some embryos will be found to be affected or at high risk for the disorder and in some, a diagnosis will not have been reached for technical reasons. These embryos cannot be used in therapeutic treatment and are therefore truly surplus to clinical requirements. They would normally be collected for confirmatory tests to check that the original diagnosis was correct (Pickering et al., 2003) or discarded. Although many of these embryos will have a specific mutation that may, in itself, be of interest in any cell line generated, some will be normal. Indeed, half of the embryos found to be male during cycles to ascertain sex to avoid X-linked disease will be genetically normal. Since many are of good progressive quality, these embryos could be useful for stem cell research. Another source of embryos are those that have been cryopreserved (Thomson et al., 1998) but are no longer needed by the patient, who also does not wish to donate them to another couple. These embryos are also usually of good quality if they survive the cryopreservation process (El-Toukhy et al., 2003). During the course of this study, a small number of surplus cryopreserved embryos were donated specifically for stem cell research and both sources of surplus embryos have been used here to attempt hES derivation.

Materials and methods

Use of spare human embryos

Use of embryos surplus to therapeutic need for research and derivation of human ES cell lines was approved by the St Thomas’s Hospital ethics committee (project number EC01/122), and licensed by the Human Fertilization and Embryology Authority (project number R0133).

Patient consent

Patients who were being seen for discussion about PGD for single gene or X-linked disorders or translocations, were asked to consider donating for stem cell research those embryos found after biopsy and testing either to be at high risk of being affected by the genetic disease or in which a diagnosis had not been reached. Each patient received general written information about research on surplus embryos at their initial visit for consideration of PGD. If they decided to pursue the PGD option, they received further written specific information at a second interview laying out the details of the intended stem cell research in accordance with the licence conditions issued by the HFEA in which it was stated that the stem cell lines would be used for research and not therapeutic purposes; that the cells may be frozen and stored indefinitely and could be used in other approved research projects; that the identity of the derived cells would be protected, and that the donor would not be informed of any specific results that might arise from studies on the cells; that they could be used for commercial gain by the institution and that the donor would not benefit from these gains; and that they may withdraw consent until the embryos were used for research purposes. The consent could be signed by the couple and witnessed at this stage or at any time before the PGD cycle.

Patients whose cryopreserved embryos were approaching the end of their legal storage period according to given consent and HFEA regulations, were approached as to whether they would be prepared to allow the now ‘surplus’ embryos to be used before this date for stem cell research rather than being discarded. Similar patient information and consent forms were used as above.

Ovarian stimulation

A standard long protocol was used to induce ovulation and controlled timing of egg retrieval (Khalaf et al., 2000). Pituitary suppression was achieved using buserelin nasal spray (Suprefact; Shire, Andover, UK), followed by ovarian stimulation with between 150 and 450 IU daily of recombinant human FSH (r-hFSH; Gonalf-F, Serono Pharmaceuticals Ltd, Feltham, Middx, UK) for the first 10–12 days. Human chorionic gondadotrophin (HCG), 10,000 IU, was administered to trigger ovulation when adequate follicular development had been demonstrated by transvaginal ultrasonography and there were at least three follicles >18 mm in diameter. Transvaginal oocyte retrieval was performed 35 h later. Intracytoplasmic sperm injection (ICSI) was used in cases where polymerase chain reaction (PCR) was to be used for diagnostic procedures to reduce the risk of contamination with spermatozoa and where sperm was of poor quality. Embryos were biopsied on day 3 of culture and usually no more than two available unaffected embryos were replaced in the afternoon of day 4. Luteal support was with progesterone pessaries (Cyclogest; Shire) 400 mg daily for 14 days. A pregnancy test was performed on day 14 post-embryo transfer.

Embryo culture, biopsy and embryo selection during PGD

Oocytes and embryos were cultured in sequential media under oil (Vitrolife, Göteborg, Sweden). Vitrolife G1 series of media was used for egg collection, fertilization, embryo culture and transfer in accordance with the manufacturer’s instructions. Following routine assisted reproduction treatment procedures (Khalaf et al., 2000), pronucleate zygotes were transferred to fresh G1.2 micro-drops on day 1, and then into G2.2 micro-drops late on day 2 for overnight culture. On day 3, the embryo biopsy procedure was performed as previously described (Handyside and Thornhill, 1998). Following biopsy of the blastomere for diagnosis, the embryos were rinsed and returned immediately to normal culture conditions as described above. The biopsied blastomeres were subject to diagnosis by fluorescence in-situ hybridization for X-linked (Munné et al., 1995) and chromosomal disorders (Scriven et
Cryopreservation and thawing of embryos from standard assisted reproduction treatment cycles

Embryos from a single patient, which were no longer needed for therapeutic treatment, were donated for stem cell research and were thawed using Medicult® solutions according to the manufacturer’s protocol (Medicult, Redhill, Surrey, UK). Remaining embryos that were either high risk/affected or were of too poor quality to be cryopreserved on day 5 were either discarded or used for research, dependent on patient wishes and consent. Embryos that had been donated for stem cell research were cultured until day 5–7 post-fertilization, depending on their rate of development.

Isolation of the inner cell mass by immunosurgery

All manipulations were carried out on heated stages and with media pre-warmed to 37°C. Embryos were checked daily and cultured until either there was developmental arrest or a clear blastocoelic cavity was evident. Unless the blastocyst had already hatched, the zona pellucida was then removed by exposure to pronase (10 mg/ml, Sigma, Dorset, UK). Subsequently, zona-free blastocysts were exposed to 30–50% anti-human serum (Sigma) in Dulbecco’s modified essential medium (DMEM) with Glutamax (Invitrogen, Paisley, UK) for 10 min, followed by a brief rinse in M2 supplemented with bovine serum albumin (BSA; Sigma) and exposure to 20% guinea pig complement (Sigma) in DMEM plus Glutamax for 5–15 min. If the blastocyst was expanded, the embryo was moved gently between all solutions with a wide bore pipette to help prevent collapse of the blastocoelic cavity. When the trophectoderm was completely lysed, the whole embryo was passed quickly through a narrow bore pipette (around 40–50 μm). If the blastocoelic cavity was still fully expanded, the trophectoderm cells were easily removed from the intact inner cell mass, if present. If the blastocoel had collapsed, this process was much more difficult, but could be achieved with perseverance. The intact inner cell mass was transferred immediately to one well of a 4-well plate (Nunclon, Biotip, UK) coated with 0.1% gelatine and mouse embryonic fibroblasts (MEF) at a concentration of 75 × 10³ per cm² in tissue culture medium suitable for embryonic stem cell culture. Tissue culture wells, culture media and MEF were prepared exactly as described by Reubinoff et al. (2000). MEF were derived from fetuses isolated from CFLP (Harlan Olac, Bicester, UK) mice or from fetuses isolated from 129/SV females crossed with C57/BL6 males (Harlan Olac, Bicester, UK).

Culture of inner cell mass

The inner cell mass was observed daily during outgrowth, left in situ for up to 15 days, and was only replated on to fresh fibroblasts if cells of stem cell-like morphology appeared during this time. Cells with stem cell-like morphology usually appeared near the centre of the colony and these were separated from surrounding endoderm by cutting with a glass needle. This growing epiblast colony was left to grow for 2–4 days after initial appearance and then removed intact if possible, without any surrounding endoderm.

Derivation of stem cell lines

After transfer to a fresh well, the growing epiblast was left to grow intact for 4–8 days with daily observation and feeding with hES medium (Reubinoff et al., 2000). When the colony had reached approximately 0.1–0.5 mm in size, it was cut into 2–10 pieces and split between 2–4 wells. This process was continued on undifferentiated colonies every 5–7 days.

Immunocytochemical characterization

After preparation for routine passage of undifferentiated colonies, several colony pieces were transferred to 24-well plates containing 13 mm² glass coverslips coated with MEF. Cells were propagated for several days in ES cell medium until the colonies reached appropriate size and were then processed for routine immunocytochemistry using standard methods (Minger et al., 1996). Briefly, cells were washed with phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde in 0.1 mol/l phosphate buffer for 30 min at 4°C. Cells were washed with Tris-buffered saline (TBS; pH 7.4, 20 mmol/l Tris, 150 mmol/l NaCl) 3 times for 15 min each at room temperature and were then incubated for 30 min in TBS containing 0.01% Triton (t-octylphenoxypolyethoxyethanol; Sigma) X-100 (TBS+). This was followed by a further 30-min incubation in 5% dried powdered milk in TBS+ in order to prevent non-specific antibody binding. Cells were incubated overnight at 4°C with primary antibodies for SSEA-3 (IgM, Developmental Studies Hybridoma Bank: DSHB, 1:10) and SSEA-4 (IgG3, DSHB, 1:10) diluted in 5% milk in TBS+. Following overnight incubation, cells were washed 3 times in TBS+ for 15 min each and then treated with isotype-specific fluorescein-conjugated, anti-mouse secondary antibodies (Vector Laboratories, Accent Park, Peterborough, UK) diluted at 1:100 in TBS+ for 1 h at room temperature. Coverslips were washed extensively in TBS and then mounted on glass slides using Vectashield containing DAPI to preserve fluorescence and to visualise all cells respectively. Images of immunoreactive colonies were obtained using a Nikon Digital Camera (DXM1200) and Lucia image analysis software (Nikon, version 4.60) integrated with a Nikon EZ00 Eclipse microscope equipped for epifluorescence. Controls for
antibody specificity included omission of the primary antibody and immunocytochemistry of SSEA-3 and 4 on mouse ES cells as well as other mouse and human non-ES cell types. In all cases, no immunoreactive cells were observed, demonstrating the specificity of the SSEA-3 and 4 antibodies for human ES cells.

Reverse transcriptase-PCR analysis

After preparation for routine passage of undifferentiated colonies, several colony pieces were washed through two 1 ml drops of PBS and transferred to a PCR tube containing 10 µl of PBS. Total RNA was isolated from individual colonies using the RNAeasy kit (Qiagen Ltd, Boundary Road, Crawley, UK) and treated with DNase (Qiagen). cDNA was synthesized simultaneously from all RNA samples using MMLV-RT Superscript II (Promega UK, Delta House, Southampton, UK). Briefly, oligo(dT)18 (1 µg) and random 10-mers (1 µg) were added to the RNA (10 µl), and the mixture was heated at 70°C, 5 min) to reduce secondary RNA structure and then cooled on ice. Dithiothreitol (10 mmol/l), 0.5 mmol/l of each dinucleotide, dATP, dCTP, dTTP, and dGTP, recombinant ribonuclease inhibitor (80 IU, RNasin), MMLV-RT (200 IU), and diethyl pyrocarbonate-treated water were added to make the final volume 20 µl and the mixture was incubated at 42°C for 50 min followed by heat inactivation of MMLV-RT at 70°C for 15 min. The cDNA was diluted 5-fold with tRNA (10 µg/ml) and used immediately in PCR reactions or stored at −20°C for future use. An aliquot of RNA was not reverse-transcribed and was diluted with tRNA and stored at −85°C. All PCR cycling conditions were as follows: denaturation at 94°C for 1 min, annealing at 55–60°C for 1 min and extension at 72°C for 2 min for 35–40 cycles. PCR for FGF4, Rex1 and Sox2 was performed using the PCR primers and the annealing temperatures previously described (Henderson et al., 2002). In addition, PCR was performed using primers for Oct4 (kindly supplied by Megan Hitchins, Institute of Child Health, London, UK) which, at an annealing temperature of 58°C, preclude the amplification of Oct4 pseudogenes; Oct4 sense primer, 5′-GAA GGT ATT CAG CCA AAC-3′; antisense primer, 5′-CTT AAT CCA AAA ACC CTG G-3′. The predicted size of the PCR product was 650 bp. Human Nanog sense primer, 5′-CAG AAG GCC TCA GCA CCT AC-3′; antisense primer, 5′-CTG TTC CAG GCC TGA TTG TT-3′. The annealing temperature used was 55°C and the predicted size of the PCR product was 211 bp. The identities of the PCR products were confirmed by sequencing.

Collection of cells for PCR during analysis of genomic imbalance by whole genome amplification and comparative genomic hybridization

Whole genome amplification (WGA) and comparative genomic hybridization (CGH) has previously been used to identify the presence of genomic imbalance in embryonic cells during PGD (Voullaire et al., 2000) and was applied here to analyse the chromosomal constitution of hES lines. Cells from both the putative King’s hES line (WT3) and a commercially available line, which had previously been karyotyped (line hES 3; ESI International, Singapore Science Park II, Singapore 117610) were subjected to analysis by CGH. Differentiated and undifferentiated cells were collected in clumps of 100–200 cells in 0.2 ml PCR tubes containing 5 µl lysis buffer (200 mmol/l KOH) and overlaid with oil. Cell lysis was achieved by incubating at 65°C for 10 min and then cooled to 4°C. The cell lysate was stored at −20°C before amplification by Degenerative Oligonucleotide Primed (DOP) PCR.

Whole genome amplification (WGA)

Each sample was neutralized with 5 µl neutralizing buffer (500 mmol/l Trizma pH, 300 mmol/l KCl, 200 mmol/l HCl). DOP PCR was employed to amplify the whole genome of the cell uniformly. A 40 µl reaction mixture was added to a final concentration of 2.5 U Taq DNA polymerase (Genosys, Pampisford, Cambridge, UK), 200 µM dNTP, 2.5 mmol/l MgCl2, 1.5 µmol/l DOP primer 5′-CGCACTCGAGNNNNNATGGTGG-3′. The reaction buffer consisted of the lysis and neutralizing buffers, giving a final concentration of 50 mmol/l Tris pH 7.4, 50 mmol/l KCl. Thermal cycling conditions were as follows: 94°C for 4 min, cycled for 8 cycles at 94°C for 1 min, 30°C for 1 min, 72°C for 3 min with a ramp of 4°C per second between the annealing and extension steps, followed by 35 cycles at 94°C for 1 min, 56°C for 1 min, 72°C initially for 3 min then extended by 10 s each cycle with a final extension of 72°C for 10 min. Male genomic DNA of 46,XY karyotype was also amplified for use in CGH.

DNA labelling and probe preparation

DOP PCR amplified DNA was ethanol precipitated, resuspended in H2O and labelled using a nick translation kit (Vysis Ltd, Richmond, UK). The time of the nick translation was varied to optimize the probe size, which was assessed by running the DNA on a 1% agarose gel. The reference DNA (PCR products from male genomic 46,XY) was labelled with Spectrum Red and the test DNA (PCR products from hES cell DNA) was labelled with Spectrum Green. The labelled probes were co-precipitated with 5µg COT-1 DNA and dissolved in hybridization mixture and H2O. The samples were denatured at 74°C for 5 min before being applied to denatured chromosome spreads on slides (Vysis Ltd).

Comparative genomic hybridization

Denatured male metaphase spreads obtained from Vysis (Vysis Ltd) were dehydrated through an alcohol series (70%, 90% and 100%) before the probe was applied and coverslips added. Hybridization of the probe proceeded in a moist chamber at 37°C for 72 h. After hybridization the slides were washed in 0.4× SSC buffer/NP-40 at 73°C for 2 min and then briefly in 2× SSC buffer/NP-40 and air dried in the dark. The slides were mounted in anti-fade medium containing diamidinophenylindole (DAPI) to counterstain the chromosomes and nuclei. The metaphase preparations were analysed using PSI MacProbe Powergene system (PSI, League City, TX, USA) – green:red ratios of >1.25:1 were indicative of a gain of material, while ratios of <0.75:1 indicated a loss.
Cryopreservation

Undifferentiated colonies were cryopreserved using the open pulled straw vitrification method exactly as described by Reubinoff et al. (2001).

Results

Culture and growth of embryos from the PGD programme

During the course of this study, surplus embryos were donated to stem cell research in eight PGD cycles, five for sex-linked disorders (36 embryos), one for spinal muscular atrophy type I (SMA; two embryos) and two for chromosomal translocations (six embryos). A total of 44 embryos were cultured from day 4 to day 5 or 6 and 24 (55%) developed to the blastocyst stage (Table 1). However, these blastocysts were of very variable morphology, with large variation in size and appearance of both inner cell mass (ICM), trophectoderm and blastocoeel. Even blastocysts of apparently excellent morphology were quite variable with regard to size and morphology of the ICM, which may have important implications for stem cell derivation (Figure 1A–F). In 10 cases a distinct ICM between 20 and 45 µm in size was isolated from blastocysts after immunosurgery. The cells in such ICM appeared healthy and tightly packed in at least one distinct area with no obvious fragmentation or cell death (Figure 1G, H). Lysed trophectoderm cells, which in some cases could not be completely removed from the ICM, could be clearly distinguished from the ICM proper (Figure 1H, I; top left in each case). In six cases, ICM isolated from blastocysts were small (15 µm or less) and of degenerate appearance, with loosely opposed cells which were often dark and granular (Figure 1I). In eight cases no discernible ICM could be identified following immunosurgery, and only lysed trophectoderm cells were isolated. The six small ICM either degenerated quickly after transfer to the mouse fibroblast feeder layer and hES medium or, after attachment to the substrate, subsequently degenerated over a period of days without further proliferation. In 7 out of 10 of the larger ICM, some cellular outgrowth was obtained after transfer. In most cases, the isolated ICM attached to the substrate within 24 h (Figure 2A) and over the next 7 days, flattened down and started to grow out (Figure 2B–D). Cells observed at this time were of very variable morphology. In some, proliferating ICM, cells were flat, with large nuclei and prominent nucleoli and in others, the structure obtained after outgrowth was rather amorphous, with no prominent single cell type (Figure 2B–D). The morphology of the outgrowth at this early stage did not seem to have any significant relationship to the appearance of stem cell-like cells at a later stage. Initial proliferation with subsequent cellular degeneration was seen in three ICM, proliferation followed by extensive cellular differentiation was observed in a further two ICM, with no cells of stem cell-like morphology seen in either case. However, in two cases, attachment, outgrowth of the ICM and initial apparent differentiation of most cells (Figure 2B–D; presumably the primitive endoderm), was followed by the appearance of cells with stem cell-like morphology (presumably arising from the epiblast), usually around the centre of the colony, around 7–14 days after initial plating (Figure 2E). These cells could be transferred to a fresh well to give rise to stem cell-like cells (Figure 2F). One of these putative lines was derived from a male embryo donated after a cycle of PGD for X-linked disorder (Becker’s muscular dystrophy) and the other from an embryo identified as at high risk for the transmittance of SMA type I.

Culture and growth of cryopreserved embryos

Fourteen embryos cryopreserved for a single patient at the pronucleate stage donated for stem cell research were thawed three or four at a time. Of these 14 embryos, two did not survive the freezing–thawing process. Of the remaining 12, eight (67%) reached the blastocyst stage and all were of reasonable morphology with discernible ICM (Table 1), although as seen in fresh PGD embryos (above), they were of variable size. All were subjected to immunosurgery and eight ICM were recovered. Three expanded in culture, and stem cell-like cells were identified in one culture after 11 days of outgrowth. These cells were subsequently isolated and passaged after 14 days.

Isolation of stem cell-like colonies and derivation of stem cell lines

In all three cases described above, where cells of stem cell-like morphology were identified, the cells of the epiblast were separated from the surrounding endoderm as an intact cellular disc if possible. This was quite difficult as the cells were only loosely opposed at this stage. It was important to avoid contaminating the stem cell-like cells with endoderm during this process, as the stem cells tended to differentiate quickly after passage when endoderm was present. Stem cell-like cells were transferred to a new well containing fresh MEF and media and grown for a further 6–10 days, during which time

Table 1. Development and fate of embryos used for stem cell research.

<table>
<thead>
<tr>
<th>Details of embryos</th>
<th>Embryos donated (n)</th>
<th>No. reaching blastocyst (%)</th>
<th>No. from which intact ICM recovered (%)</th>
<th>No. of ICM which outgrew (%)</th>
<th>No. from which stem cell-like cells isolated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh (from PGD) (%)</td>
<td>44</td>
<td>24 (55)</td>
<td>16 (36)</td>
<td>7 (16)</td>
<td>2 (5)</td>
</tr>
<tr>
<td>Cryopreserved (%)</td>
<td>14</td>
<td>8 (57)</td>
<td>8 (57)</td>
<td>3 (21)</td>
<td>1 (7)</td>
</tr>
<tr>
<td>Total (%)</td>
<td>58</td>
<td>32 (55)</td>
<td>24 (41)</td>
<td>10 (17)</td>
<td>3 (5)</td>
</tr>
</tbody>
</table>
they proliferated without obvious differentiation (Figure 3A–C). One colony was very slow growing with densely packed cells and a dome like structure (Figure 3B). This line was transferred between wells for seven passages, but after 60 days, no significant cellular proliferation was observed and the line was thus discarded. The other two putative lines were almost identical in morphology but were flatter, with tightly packed cells with large nuclei and dense nucleoli which are typical of stem cell lines (Figure 3D–F). Initial colonies were passaged by cutting into three to six pieces and these clumps of cells were transferred to new wells. Usually, two to five colonies of undifferentiated appearance were generated by this process, which continued to proliferate upon passage. In subsequent passages during expansion of the line, clumps of cells from undifferentiated colonies were transferred to new wells and grown for 4–8 days before being split again. Around 30% of colonies showed signs of differentiation on passage, but this was very variable according to the size of clump transferred and the morphology of the originating colony. These putative stem cell lines were easy to passage and maintain. Unfortunately, one line was lost after 10 passages (>60 days in continuous culture) due to contamination of an undetermined nature, before characterization or cryopreservation had been performed. The remaining line, which had very similar morphology and growth characteristics was cryopreserved by vitrification and characterized as detailed below.

**Characterization of putative stem cell lines**

The putative hES cell line (WT3) was assessed for expression of ES cell-specific proteins and genes by immunocytochemistry and RT-PCR, respectively. Colonies (passage 6) propagated on MEF in hES medium without obvious differentiation expressed cell surface proteins...
characteristic of undifferentiated hES and EC cells, including SSEA-3 (Figure 4A) and SSEA-4 (Figure 4B). Genetic analysis of similar colonies revealed expression of Oct-4, Rex-1, Sox-2, FGF-4 and Nanog (Figure 4C).

Discussion

The first embryonic stem cells to be identified were derived from rabbit blastocysts in the early 1960s by Edwards and colleagues, who were quick to acknowledge the potential of these cells in tissue replacement therapy (Cole et al., 1966; reviewed in Edwards, 2001). Despite these early successes, it was over 30 years later that the first fully characterized pluripotent hES cell line was reported by Thomson et al. (1998). Reubinoff followed this 2 years later with a report of the derivation and differentiation of hES lines (Reubinoff et al., 2000), building on the meticulous work of Bongso and colleagues, who had for over a decade been developing coculture systems to enable the study of the human blastocyst during extended growth in vitro as well as studying the growth and behaviour of isolated inner cell masses (Bongso et al., 1992, 1994; Bongso and Fong, 1993).

Under license from the Human Fertilization and Embryology Authority (HFEA, UK), attempts have also been made to derive hES cell lines in this study, this time using a novel source of fresh embryos that could not be used in treatment during PGD cycles. As a comparison, a small number of
**Figure 3.** Phase contrast micrographs of various ES cell colonies during derivation. (A) Example of a colony from hES line 1, passage 6. (B) Example of a colony from hES line 2, passage 3. Note unusual domed morphology. Cells in this line grew very slowly and so the line was discarded. (C) Example of colony from hES line 3 (WT3), passage 5. Note distinctive colony edge with surrounding MEF. (D) Colony from WT3 passage 6. Note outgrowth of hES cells from central ‘button’. (E) and (F) Higher magnification photographs of colonies from line WT3, passage 6 to show conspicuous small cell size and large nucleoli, characteristic of hES cell morphology. Original magnifications – A, C, D: ×100; B, E: ×200; F: ×400
cryopreserved embryos that were surplus to therapeutic requirements and had been donated specifically for use in stem cell research were also used. To date, three sets of hES cell-like cells have been derived from a total of 58 human embryos. One of the stem cell populations, although composed of morphologically hES-like cells, proliferated very slowly and seemed to arrest in culture after approximately 2 months and was subsequently discarded. However, two additional sets of hES-like stem cells proliferated avidly and were similar in morphology to cell lines isolated by Thomson et al. (1998), Reubinoff et al. (2000) and Lanzendorf et al. (2001). The initial putative cell line was lost to contamination prior to characterization, but one cell line (WT3) was also characterized by immunocytochemistry and in common with published hES cell lines, expressed both SSEA-3 and SSEA-4 cell-surface antigens. Both of these glycolipid-linked proteins are uniquely expressed on human ES and embryonal carcinoma (EC) cells, but their expression is significantly down-regulated in hES cells upon differentiation (Draper et al., 2002).

In addition to the presence of ES cell-specific proteins, genetic analysis demonstrated that the putative hES cell line expressed a number of genes shown to be specifically expressed within the preimplantation embryo/inner cell mass of the developing blastocyst or in pluripotent ES cell lines by genetic profiling of different stem cell populations (Ramalho et al., 2002). This included expression of Oct-4, a POU transcription factor required for the generation of pluripotent hES cells from the inner cell mass (Nichols et al., 1998; Niwa et al., 2000), Rex-1, an acidic zinc finger transcription factor shown to be a specific regulatory target of Oct-4 in mouse ES cells (Ben-Shushan et al., 1998), and PFG-4, a fibroblast growth factor present within the ICM (Rappolee et al., 1994) and another transcriptional regulatory target of Oct-4 in ES cells (Yuan et al., 1995). The putative hES cell line generated in the laboratory also expresses Nanog, a homeodomain protein recently shown by Chambers et al. (2003) and Mitsui et al. (2003) to be obligatory for maintaining pluripotency in mouse ES cells. So far as is known, this represents the first demonstration of the expression of Nanog mRNA in a putative hES line. Therefore, the WT3 cell line generated fulfills many of the molecular and cellular characteristics of an hES cell population.

To prove that these cells are fully pluripotent, it will be necessary to demonstrate their ability to contribute to all three germ layers, endoderm, ectoderm and mesoderm. This is normally achieved by the formation of embryoid bodies in vitro followed by histochemistry and morphological assessment or by injection under the kidney testis capsule of SCID mice to demonstrate teratoma formation. In addition, several samples of differentiated and undifferentiated hES cells from the WT3 line were tested for genomic imbalance using CGH (Voulaire et al., 2000). During CGH, PCR samples from test DNA and reference DNA of known genotype are labelled with a red or green fluorochrome and the samples are then competitively hybridized to a normal male chromosome spread. Any excess red or green signal on an individual chromosome indicates genomic balance. Although genomic imbalance could not be identified in the WT3 hES samples subjected to CGH in this study, a full karyotype still needs to be carried out to confirm that the cell line is indeed of normal diploid phenotype. These studies are currently under way.

On the basis of origin, cell morphology and molecular characterization, the cell lines described here are believed to be the first reported hES cell lines to have been derived in the UK, under the strict legislative framework set out by the HFEA (HFEA, 1999). Under these regulations a licence is required for any project of research that utilizes human embryos, and a strict code of practice is in force for the derivation of stem cell lines including a requirement to deposit a sample of the cells in the MRC national stem cell bank. To derive one of these cell lines, a novel and ethical source of embryos, that are clearly unsuitable for clinical use but can yield blastocysts of extremely high quality, has been used. The first putative line, which was subsequently lost to bacterial contamination before characterization, was derived from a male embryo that had been generated as part of a PGD cycle for X-linked disease (Braude et al., 2002). This embryo was deemed at high risk for the transmission of Becker’s muscular dystrophy and could not be replaced, but was a high quality, progressive blastocyst with a clear ICM on the day of immunosurgery. This approach to the generation of stem cell lines could result in several lines carrying homozygous mutations in key genes involved in common genetic disorders such as cystic fibrosis (CF), spinal muscular atrophy (SMA) and Huntington’s disease, all disorders for which PGD has been applied in various centres around the world (Geraedts et al., 2002; Kuliev and Verlinsky, 2002; Sermon, 2002).

Such lines could provide valuable in-vitro tools to investigate cell–cell interactions, disease progression and the efficacy and toxicity of drug therapies at the cellular and molecular level, both before and after differentiation into specific cell types. Embryos which become available for stem cell derivation may fall into one of three categories: a) embryos in which a specific diagnosis is not available, but which are considered as high risk for transmitting a particular disease and are therefore unsuitable for replacement. These include those embryos diagnosed as male following PGD for X-linked disorders (only 50% are affected by the disease); b) a variable proportion (up to 30%) of embryos for which a diagnosis could not be reached due to failure of the diagnostic test in the biopsied cell; c) a proportion of embryos (up to 10%) classified as homozygous affected for a single gene disorder which may, in fact, be heterozygous. In PGD for single gene disorders, embryos can sometimes be classified as homozygous affected due to allele drop out, a phenomenon that is common in single cell PCR (Findlay et al., 1995; Ray et al., 1996). In the case of an autosomal recessive disorder such as SMA or CF, stem cells arising from such an embryo would be essentially normal and therefore useful for stem cell research but could also be utilized within the PGD unit as a convenient source of heterozygous cells for assay development and quality control experiments in the absence of a transformed cell line carrying this mutation.

In previous reports, hES lines have been derived using both fresh and cryopreserved embryos that were surplus to therapeutic requirements following assisted reproduction treatment procedures (Lanzendorf et al., 2001; Reubinoff et al., 2000; Thomson et al., 1998). The results reported here confirm that cryopreserved embryos are a useful source of
Figure 4. Characterization of line hES line WT3 including biochemical marker expression (A and B) and RT-PCR analysis (C) of individual colonies. (A) ES cell colony stained with antibody recognizing the SSEA-3 epitope. (B) ES cell colony stained with antibody recognizing the SSEA-4 epitope. (C) PCR products obtained using primers specific for Oct4, FGF4, Rex1, Sox2, Nanog and β-actin.

Figure 5. Representative comparative genomic hybridization (CGH) profile obtained following hybridization of labelled DOP-PCR products from King’s putative cell line WT3 (as the test sample) with normal male DNA (as the reference sample). Note the deviation to the left of the trace for chromosome Y and deviation to the right for trace for chromosome X indicating the likelihood that these cells were derived from a female embryo. No significant divergence is seen for the other chromosomes. Enhancement at the ends of chromosomes is frequently observed and is regarded as artefactual.
embryos for stem cell research, with 1/14 (7%) of the embryos thawed giving rise to a putative hES cell line. However, on the basis of this study, PGD appears to be an additional and important source of fresh embryos suitable for stem cell research, with 2/44 (5%) donated embryos giving rise to a putative hES line, suggesting that the procedure is reasonably efficient with such material. Use of fresh embryos from PGD for hES cell derivation may obviate some of the concerns regarding fresh embryos from infertile couples being used for this purpose.

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