**Derivation of a diploid human embryonic stem cell line from a mononuclear zygote**

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**BACKGROUND:** IVF occasionally produces aneuploid zygotes with one or three pronuclei (PN). Routinely, these zygotes are discarded. The aim of this work was to establish human embryonic stem cell (hESC) lines from blastocysts resulting from abnormal fertilization. **METHODS:** Abnormally fertilized zygotes were cultured to the blastocyst stage and, following zona pellucida digestion, zona-free blastocysts were placed on a mouse feeder layer. Culture of hESCs was carried out as described earlier. **RESULTS:** Six out of the nine developing blastocysts attached to the feeder layer. One hESC line, originating from a mononuclear zygote following ICSI, was successfully derived. This line displayed typical phenotype and embryonic surface markers, and exhibited the potential to develop into all three embryonic germ layers both in vitro (by embryoid body formation) and in vivo (teratoma generation). Genetic examination revealed normal diploid karyotype and heterozygotic appearance for metachromatic leukodystrophy (MLD). **CONCLUSION:** This method, which requires neither immuno nor mechanical removal of the trophectoderm, may facilitate the derivation of hESC lines in general, and those from abnormal embryos in particular. Furthermore, it is shown that aneuploid zygotes can be used as a source for normal hESC derivation and hold the potential to generate aneuploid hESC lines for research purposes.

**Key words:** aneuploid zygote/derivation/differentiation/embryonic stem cells

**Introduction**

Normal human zygotes consist of two pronuclei representing each parent. However, IVF often results in abnormal zygotes with one or three pronuclei (PN). The high percentage of diploid embryos which was reported to develop from IVF 1PN zygotes (Munne *et al*., 1993) drops to 10–30% following ICSI (Sultan *et al*., 1995; Staessen and Van Steirteghem, 1997).

At the blastocyst stage, the embryo forms an inner cell mass (ICM) capable of forming a wide range of cell types of the body, and an outer trophectoderm, which is committed to form part of the placenta (Winkel and Pedersen, 1998). Isolation and seeding of the ICM on an appropriate feeder layer may generate human embryonic stem cells (hESCs) (Thomson *et al*., 1998; Reubinoff *et al*., 2000). Such isolation can be accomplished by removal of the trophectoderm either mechanically (Amit and Itskovitz-Eldor, 2002) or by immunosurgery (Thomson *et al*., 1998; Reubinoff *et al*., 2000; Lanzendorf *et al*., 2001; Amit and Itskovitz-Eldor, 2002). All hESCs have the ability to self-renew perpetually in culture and maintain undifferentiated phenotype and normal karyotype. They are pluripotent, i.e. capable of developing into all three primary germ layer derivatives, namely ectoderm, mesoderm and endoderm, both in vitro and in vivo [embryoid body (EB) and teratoma formation, respectively] (Draper and Andrews, 2002). To date, blastocysts used for hESC derivation have been obtained from donated normal embryos (Thomson *et al*., 1998; Reubinoff *et al*., 2000; Lanzendorf *et al*., 2001; Amit and Itskovitz-Eldor, 2002) or from poor-quality discarded embryos (Mitalipova *et al*., 2003). This work aimed at deriving hESCs from non-usable aneuploid zygotes. As it was reported that mouse ES cells can be derived by simply culturing the blastocyst on the feeder layer (Nagy *et al*., 2003), this was also examined in the human.

**Materials and methods**

**Blastocyst culture**

Discarded zygotes were donated to this study by couples undergoing IVF at Rambam Medical Center who signed consent forms. Zygotes (n = 60) were cultured to the blastocyst stage according to our IVF laboratory standard protocol: drops under oil using Cook media (Cook, Queensland, Australia) for insemination, growth and blastocyst stage (Cook IM, GM and BM, respectively).

**hESC derivation and cultivation**

After zona pellucida digestion by Tyrode’s acidic solution (Sigma, St Louis, MO), whole blastocysts were placed on mitotically inactivated mouse embryonic fibroblasts (MEFs). Cells were grown, passaged,
frozen and thawed as previously described (Thomson et al., 1998; Amit and Itskovitz-Eldor, 2002).

EB and teratoma formation, genetic characterization and histological sections

All hESC characterizations were done as previously described (Amit et al., 2003). Mutation of metachromatic leukodystrophy (MLD) was detected using DNA which was isolated from cells of the newly derived cell line, by mutation-specific PCR. The analysis was performed by the Genetic Unit at Hadassah University Hospital, Ein-Karem, Jerusalem, Israel and by the Developmental Biology Unit, Department of Obstetrics and Gynaecology, Rambam Medical Center, Haifa, Israel.

Immunofluorescence and confocal microscopy

Cultured colonies or attached EBs were fixed in situ with 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS; Gibco, San Diego, CA) for 30 min at room temperature. After blocking with 10% serum, the cells were stained with one of the following primary antibodies: anti-stage-specific embryonic antigen 3 (SSEA3) or 4 (SSEA4), anti-tumour rejection antigen (TRA) 1–60 and 1–81, all kindly provided by Professor P. Andrews, University of Sheffield, UK), anti-CD31 (Dako, Denmark) and anti-cytokeratin 17 and anti-tubulin βIII isoform (both from Chemicon International, Temecula CA). Cells were then rinsed three times with PBS (Gibco) and incubated for 30 min with a suitable fluorescein isothiocyanate (FITC)- or Cy3-conjugated secondary antibody (Sigma). 4',6-Diamidino-2-phenylindole (DAPI) or propidium iodide (PI) (Sigma) was added (1:1000) to the last rinse. The immunolabelled cells were examined using either fluorescence microscopy (Carl Zeiss, Jena, Germany) or a confocal laser scanning system (BioRad Laboratories Ltd, Hertfordshire, UK).

Results

Blastocyst culture

Applying our standard protocol for blastocyst culture, most aneuploid zygotes failed to develop into blastocysts. The nine out of 60 zygotes that developed into blastocysts consisted of seven 3PN and two 1PN. These zygotes showed either typical blastocyst morphology or irregular blastocyst morphology, i.e. large peripheral cells and an unclear ICM.

hESC line derivation and cultivation

Zona pellucida was digested and blastocysts were placed on a mitotically inactivated mouse feeder layer (Figure 1A). Within 24 h, six out of the nine blastocysts attached to the feeder layer (Figure 1B). The trophoderm was immersed within the feeder layer and ceased to proliferate. In contrast, after several days, small tightly packed cells began to proliferate from four clumps. Each of the small colonies was mechanically dissociated, divided into two and replaced on a fresh feeder layer. A few days later, the same procedure was repeated. Most isolated ICM clumps died out after two or three passages, leaving a single ICM colony which continued to proliferate and propagate. Consequently, one hESC line was established (Figure 1C). The line, designated I9, exhibited normal growth rate, was passaged for >40 passages and survived freeze and thaw cycles.

hESC line characterization

Characterization of the derived line was conducted according to the following parameters.

Morphology. The I9 line continued to proliferate and maintained a morphology typical of hESC colonies, i.e. round colonies with sharp edges (Figure 2A), in which the spaces between cells are clear (Figure 2B). Single cell morphology showed a high nucleus to cytoplasm ratio with the presence of at least two nucleoli (Figure 2C).

Genetic analyses. Karyotype analyses were performed on 66 cells from passages 27 and 29. Both analyses revealed normal human karyotype (46,XX). Two examples of the examined chromosomes are shown in Figure 3. As the line was donated by a patient undergoing preimplantation genetic diagnostic (PGD) treatment, analysis for the specific MLD mutation was also performed. DNA was extracted at passages 10 and 24. The line was found to be heterozygous to the disease.

Embryonic markers. The hESC line grown for 32 passages was found to stain positive for typical primate ES cell surface markers (Thomson et al., 1998) SSEA4, TRA-1–60 and TRA-1–81 (Figure 4), and weakly positive for SSEA3.

Figure 1. De novo hESC line derivation. (A) An abnormal blastocyst after zona pellucida digestion placed whole on an inactivated feeder layer. (B) Attachment occurred within 24 h. (C) An hESC colony derived from the blastocyst. Bar = 100 μm.
EB formation in vitro. Similarly to other hESCs, once removed from its feeder layer and cultured in suspension (Itskovitz-Eldor et al., 2000) (Figure 5A), line I9 formed EBs, including cystic ones (Figure 5B). Stem cells within these EBs differentiated into various cell types (Figure 5C).

The developmental potential of line I9 was examined by expression of representatives of the three germ layers in 10- to 15-day-old EBs. Formation of blood vessel-like structures (Figure 6A), neuron generation (Figure 6B) and epithelial cell sheets expressing low molecular weight cytokeratins (Figure 6C) were indicative of mesoderm, ectoderm and endoderm, respectively.

Teratoma generation in vivo. Injection of undifferentiated I9 cells into the hind limb muscle of a SCID-beige mouse resulted in the generation of a teratoma which possessed representatives of all three germ layers (Figure 7).

Discussion
Blastocysts developed from aneuploid zygotes may exhibit atypical morphology in which it is difficult to identify an ICM zone, with a clear distinction between the ICM and trophoectoderm. Using a method where whole blastocysts were placed on inactivated MEFs produced a successful attachment rate (six out of nine) and the proliferation of pluripotent clumps. This method has already been used in the isolation of mouse ICM (Nagy et al., 2003), and is technically simpler than immunosurgery or mechanical removal of the trophoectoderm. It is feasible in human blastocysts and may...
facilitate the derivation of hESC lines from abnormal and normal embryos. However, a more in-depth comparison between the different techniques of ICM isolation should be performed in order to be able to determine which is the most efficient for the derivation of hESC lines. A new hESC line, line I9, was successfully generated from a mononuclear zygote. This line expressed specific embryonic markers and could easily differentiate into derivatives of the three embryonic germ layers. Although this line originated from a mononuclear zygote, it was found to have normal diploid karyotype (46,XX). It is well established that following ICSI, 10–30% of mononuclear zygotes can develop into normal blastocysts, probably due to normal fertilization followed by asynchronous formation of PN (Sultan et al., 1995; Staessen et al., 1997). Hence, these otherwise discarded zygotes may be used as an available source for the derivation of diploid hESC lines and may generate aneuploid hESC lines for research.

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References


Figure 5. Spontaneous EB formation. hESCs removed from their feeder and grown in suspension formed EBs. (A) Three-day-old EBs. (B) A cyst developed in more mature EBs (9 days old). (C) A histological section stained with H&E revealed various cell shapes including clear cysts (arrow) and other voids (arrowheads).

Figure 6. Developmental potential in vitro. (A) CD31+ cells show three-dimensional network formations, vascular-like channels within 11-day-old EBs (×600). (B) Neuron networks were visible with the anti-tubulin βIII isoform (×800) (nuclei stained red with PI). (C) Endodermal epithelium, stained with anti-cytokeratin 17, showed typical sheet organization with low molecular weight cytokeratin distribution (×800).

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Figure 7. Developmental potential in vivo. Histological sections of the resultant teratoma from line I9 at passage 37 revealed representatives of all three germ layers, such as (A) stratified epithelium (ectoderm) and cartilage tissue (mesoderm) and (B) secretory glands (endoderm). Bar = 100 μm.