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Laser-assisted derivation of human embryonic stem cell lines from IVF embryos after preimplantation genetic diagnosis

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BACKGROUND: Human embryonic stem cells (hESCs) suitable for future transplantation therapy should preferably be developed in an animal-free system. Our objective was to develop a laser-based system for the isolation of the inner cell mass (ICM) that can develop into hESC lines, thereby circumventing immunosurgery that utilizes animal products. METHODS: Hatching was assisted by micromanipulation techniques through a laser-drilled orifice in the zona pellucida of 13 abnormal preimplantation genetic diagnosed blastocysts. ICMs were dissected from the trophectoderm by a laser beam and plated on feeders to derive hESC lines. RESULTS: eight ICMs were isolated from nine hatched blastocysts and gave rise to three hESC lines affected by myotonic dystrophy type 1, hemophilia A and a carrier of cystic fibrosis 405+1G>A mutation. Five blastocysts that collapsed during assisted hatching or ICM dissection were plated whole, giving rise to an additional line affected by fragile X. All cell lines expressed markers of pluripotent stem cells and differentiated *in vitro* and *in vivo* into the three germ layers. CONCLUSIONS: These hESC lines can serve as an important model of the genetic disorders that they carry. Laser-assisted isolation of the ICMs may be applied for the derivation of new hESC lines in a xeno-free system for future clinical applications.

Keywords: laser; preimplantation genetic diagnosis; human embryonic stem cells; derivation; inner cell mass

Introduction

Human embryonic stem cells (hESCs) (Thomson *et al*., 1998; Reubinoff *et al*., 2000) can potentially proliferate indefinitely in culture and give rise to any cell type. Therefore, hESCs may serve as a renewable source of cells for future transplantation therapy. To exploit the potential of hESCs for regenerative medicine, they should preferably be derived and propagated in an animal-free culture system. However, all but one (Ellerstrom *et al*., 2006) of the reported hESC lines to date were developed within culture systems that included animal products.

The derivation process of most of the reported hESC lines included the isolation of the inner cell mass (ICM) from the trophectodermal (TE) cells of the blastocyst. Few hESC lines were derived after plating intact blastocysts on feeders, without isolating the ICM (Heins *et al*., 2004). At present, it is unclear which approach is more efficient (Hovatta, 2006).

Immunosurgery has been most commonly used for the selective lysis and removal of the TE cells from the ICM (Thomson *et al*., 1998; Reubinoff *et al*., 2000; Cowan *et al*., 2004). However, immunosurgery utilizes animal-derived products such as anti-human antiserum and guinea pig complement. These reagents may include animal pathogens and molecules (Martin *et al*., 2005) that may contaminate the ICMs and the hESCs derived from them. Therefore, immunosurgery should preferably be avoided in the process of deriving hESC lines for future clinical applications.

To circumvent the use of immunosurgery, mechanical dissection (Amit and Itskovitz-Eldor, 2002; Hovatta, 2006) or chemical dissolution of the TE layer (Ellerstrom *et al*., 2006) were used to isolate the ICMs. Mechanical dissection is a crude method, which is dependent on the operator's technical skills. Chemical dissolution of the TE layer by acid tyrode may damage the cells of the ICM due to the acidification of the medium.

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Dissection by laser may serve as an alternative approach to chemical or mechanical removal of the TE cells. Laser drilling is currently the preferred method for creating an opening in the zona pellucida of cleavage-stage embryos for blastomere biopsy for preimplantation genetic diagnosis (PGD). In a comparison of laser drilling with acid tyrode dissolution of the zona pellucida (Joris *et al*., 2003), the laser approach resulted in more intact blastomeres.

Laser-assisted dissection of ICMs from murine blastocysts and subsequent derivation of mouse ESCs was recently reported (Cortes *et al*., 2006; Tanaka *et al*., 2006). However, the use of laser for the derivation of ESCs from human blastocysts was not reported in peer reviewed scientific literature. In the present study, a non-contact laser was used both for assisted hatching and dissection of the ICM from the TE cells of human blastocysts. Eight ICMs were successfully isolated from nine genetically abnormal PGD blastocysts, and three hESC lines were derived from them. An additional hESC line was developed from five PGD abnormal blastocysts that collapsed during the micromanipulation procedures and were therefore plated whole on feeders.

Thus, our results show that laser dissection facilitates the isolation of the ICM without the use of animal products and may be useful for developing hESCs for potential future clinical applications. Furthermore, the new hESC lines reported here can serve as a unique, important model for the study of the pathophysiology and the development of new therapies for the genetic disorders that they carry.

Material and Methods

Human embryos

PGD embryos were donated by couples undergoing IVF treatment at the IVF unit of the Hadassah Medical Center of The Hebrew University. Embryos that were not diagnosed as genetically normal were recruited for the study subject to informed consent of the couples. The study was approved by the ethical committee at the Hadassah Medical Center as well as the Israeli Ministry of Health National Helsinki Committee for Genetic Research in Humans.

PGD procedure

ICSI was used to fertilize mature oocytes that were further cultured for 72 h in P1 medium supplemented with 10% serum substitute supplement (SSS) (both from Irvine Scientific, Santa Ana, CA, USA) at 37°C in 5% CO_2, 5% O_2 atmosphere, or in G1 medium (Vitrolife, Kungsbacka, Sweden) in 6% CO_2, 5% O_2. Blastomere biopsy was performed on embryos that contained at least four blastomeres. The zona pellucida was perforated by laser pulses (200 mW × 0.3 ms) from non-contact Zilos-tk^{TM} infrared laser system (Hamilton Thorne Biosciences, Beverly, MA, USA). One or two cells were removed for fluorescence *in situ* hybridization or PCR analysis. For PCR analysis, the cells were washed five times in 4% phosphate-buffered saline (PBS, supplemented with 4% SSS both from Irvine Scientific), transferred to microcentrifuge tubes (one cell per tube) and were delivered on dry ice to the lab of the Department of Human Genetics in the Hadassah Medical Center where they were individually diagnosed.

Genetic diagnosis of single blastomeres

DNA was extracted as described by Verlinsky *et al*. (2001). Multiplex fluorescent PCR (F-PCR) was used to analyse allele-specific mutations (when applicable) and linked polymorphic markers (Harper *et al*., 2002).

The diagnosis of myotonic dystrophy type 1 (DM1) was based on linkage analysis using three polymorphic markers linked to the *DMPK* gene; APOC2, D19S219 and D19S412. These markers were informative in the family. With regard to cystic fibrosis (CF), the two familial mutations were 405+1G>A and 2751+1insT. These mutations were analysed by allele specific PCR. In addition, three polymorphic markers CFTRint1, NUR and CFTR3 from within the *CFTR* gene were analysed.

The diagnosis of hemophilia A was based on linkage data from an affected child and parents, using the polymorphic markers DXS8087, DXS1073 and intragenic marker int13 (factor VIII). PCR of *AMELY*/*AMELX* was used to distinguish XX and XY blastomeres. Four polymorphic markers $(CA)_n$rep from within the *FMR1* gene and flanking markers, DXS8091, DXS1193 and IDS were used for the diagnosis of fragile X (FRAXA).

Confirmation of the diagnoses in the hESC lines

The diagnosis of DM1 was confirmed in the hESCs by linkage analysis as described above for PGD and by triplet repeat primed PCR (TP PCR) method to confirm the expansion mutation $(CAG)_n$ according to published protocols (Warner *et al*., 1996). PCR analysis of the specific mutations and the polymorphic markers as above was used to confirm the diagnosis of CF. With regard to hemophilia A, linkage analysis was used as described for PGD. Fragile X diagnosis was confirmed by linkage analysis as described for PGD and by southern hybridization after digestion of genomic DNA with EcoR1 and Eag1 and hybridization with the probe StB12.3 (Rousseau *et al*., 1991).

Embryo culture and laser-assisted ICM isolation

After blastomere biopsy, the embryos were transferred into Blastocyst medium supplemented with 10% SSS (both from Irvine Scientific) or G2 medium (Vitrolife). Blastocyst morphology was scored according to Gardner and Schoolcraft (1999).

Dissection of the ICM was performed under oil in the medium and culture dishes (Falcon cat. no 353653, Becton Dickinson, Franklin Lakes, NJ, USA) that were used for blastocyst culture.

In cases of unhatched or partially hatched blastocysts the first step was the removal of the blastocysts from the zona pellucida (assisted hatching). The blastocysts were secured to a holding pipette (Cook, Limerick, Ireland, cat. no. K-HPIP-3320) with the hole that was made in the zona pellucida for blastomere biopsy being positioned at 3 o'clock. An embryo biopsy pipette with an inner diameter of 35 μm (Cook cat. no. K-EBPH-3525) was used to assist the hatching of the blastocysts from the zona pellucida. In cases of non-hatching blastocysts, the biopsy pipette was introduced through the hole in the zona pellucida to secure the TE cells adjacent to the hole, followed by gentle pulling of the blastocysts from the zonal cavity. When some of the TE cells had already spontaneously hatched, they were gently secured to the pipette and the blastocysts were gently pulled out of the zona pellucida as above.

For ICM isolation the hatched blastocysts were re-secured to the holding pipette with the ICM positioned at 3 o'clock. The ICM with a few TE cells attached to it was gently drawn into the biopsy pipette and pulled gently away from the TE cells. The ICM was detached by 20–30 infrared laser pulses (200 mW × 0.5 ms, Zilos-tk^{TM}, Hamilton Thorne Biosciences). Special attention was paid to direct the laser beam far enough from the ICM to prevent heating and damage to the ICM.

In cases of collapse of the blastocysts during assisted hatching by micromanipulation or ICM dissection and therefore causing difficulty

in identifying the ICM, the whole blastocyst was plated on feeders for further derivation of hESCs.

Derivation of hESCs

The isolated ICMs or whole blastocysts were plated on Mitomycin-C inactivated (10 μg/ml for 2.5 h; Sigma, St Louis, MO, USA) human foreskin fibroblasts (a gift from M. Revel, Rehovot, Israel). For the preparation of feeder layers, 10^5 mitotically inactivated feeders were plated in center-well tissue culture dishes (Falcon, cat. no. 353037, Becton Dickinson) pre-coated with 0.1% gelatin (Sigma) in hESCs culture medium at 37°C in 5% CO_2, 4% O_2 atmosphere (Ezashi *et al*., 2005). The hESC medium comprised Knockout Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% Knock-Out SR, 2 mM L-glutamine, 1% nonessential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1 mM β-mercaptoethanol (all from Invitrogen Corporation, Grand Island, NY, USA), supplemented with 4 ng/ml basic fibroblast growth factor (bFGF) (PeproTech Inc., Rocky Hill, NJ, USA).

After 5–8 days, small tightly packed cells were dissected mechanically from the ICM or whole embryo outgrowths and plated on fresh feeders. Colonies of cells resembling hESCs gradually appeared within a few passages. Routine passaging of putative hESCs was performed by mechanical dissociation of the colonies into small clusters and plating on fresh feeders. Clusters of early passage hESCs were cryopreserved by vitrification according to our published protocol (Reubinoff *et al*., 2001) or by conventional slow cooling in 90% fetal calf serum (FCS, Biological Industries, Kibbutz Beit Haemek, Israel) and 10% dimethylsulfoxide (Sigma). For the development of bulk cultures and a large number of cells, the hESCs were passaged by enzymatic dissociation. The hESC colonies were dissociated into small clusters by incubation for up to two hours with 1 mg/ml collagenase type IV solution (Invitrogen). Bulk passaging was performed up to 10 weeks.

Characterization of hESCs

Immunofluorescence staining

For demonstrating the expression of Oct-4, hESC colonies were triturated into small clusters, plated on glass coverslips pre-treated with poly-D-lysine (30–70 kDa, 10 μg/ml; Sigma) and laminin (4 μg/ml; Sigma) and were incubated in hESC medium for up to 24 h at 37°C in 5% CO_2, 4% O_2 atmosphere. The cells were subsequently washed with PBS, fixed with 4% paraformaldehyde (PFA) and incubated with mouse monoclonal anti-human Oct-4 antibody (1:50; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Immunostaining with rat monoclonal anti-human SSEA-3 (1:50), mouse monoclonal anti-human SSEA-4 (1:100), TRA-1-60 (1:50) and TRA-1-81 (1:50) antibodies (all from Chemicon International, Temecula, CA, USA) was performed on intact colonies cultured on foreskin feeder layer after fixation with 4% PFA. Polyclonal goat anti-mouse immunoglobulins (Ig) conjugated to fluorescein isothiocyanate (1:50; Dako Corporation, Carpinteria, CA, USA) or affinity purified goat anti-mouse IgG conjugated to Cy3 (Jackson Laboratories, West Grove, PA, USA) were used for localization of anti-SSEA-4, TRA-1-60 and TRA-1-81 antibodies. Mouse anti-rat IgM conjugated to R-phycoerythrin (1:20; Southern Biotechnology Associates Birmingham, AL, USA) was used for detection of anti-SSEA-3. Mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories Inc., Burlingame, CA, USA) was used for nuclei counterstaining and the specimens were visualized with Nikon E600 fluorescent microscope.

Alkaline phosphatase activity detection and karyotyping

Vector Red Alkaline Phosphatase substrate kit I (Vector Laboratories Inc.) was used for detection of alkaline phosphatase activity within intact colonies on feeders according to the manufacturer's instructions.

For karyotype analysis, hESC colonies that were expanded with collagenase type IV were incubated for 2–4 h with 0.2–0.3 μg/ml Demecolcine (Sigma). The hESC colonies were then removed from the feeders, dissociated with 0.05% EDTA (Biological industries), centrifuged at 170 g for 5 min, re-suspended in 0.075 M KCl (Sigma) and incubated for 10 min in 37°C, followed by fixation with 3:1 methanol/acetic acid. The karyotype of 20 metaphases was analysed using the G-banding method.

Analysis of pluripotency after differentiation in vitro

Colonies of undifferentiated hESCs were removed from the feeder with 1 mg/ml collagenase type IV and cultured in suspension as embryoid bodies (Ebs) or neurospheres. For the development of EBs, the hESC free-floating clusters were cultured 3 weeks in DMEM supplemented with 20% FCS, 2 mM L-glutamine, 1% nonessential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1 mM β-mercaptoethanol. Neurospheres were developed as previously described (Itsykson *et al*., 2005). Briefly, the hESC clusters were cultured 3 weeks in DMEM/F12 (1:1) (Invitrogen) supplemented with B-27 (1:50) (Invitrogen), 2 mM L-Glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 20 ng/ml bFGF and 500–700 ng/ml rm-Noggin (R&D Systems Inc., Minneapolis, MN, USA).

After the 3 weeks of suspension culture, the EBs and the neurospheres were partially dissociated by triturating or by mild trypsin digestion and plated on glass coverslips pre-treated with10 μg/ml poly-D-lysine (Sigma) and 4 μg/ml laminin (Sigma), and cultured for an additional 2–7 days in the same culture media. They were fixed with 4% PFA and then stained with mouse monoclonal anti-human β-tubulin isotype III (1:2000; Sigma), mouse monoclonal anti-human desmin (1:20; Dako), and mouse monoclonal anti-human sox-17 (1:50; R&D Systems Inc.). The secondary antibodies were as described above.

Analysis of pluripotency in vivo

Pluripotency of hESCs was assessed by teratoma formation in non-obese diabetic severe combined immunodeficient (NOD SCID) mice (Harlan, Jerusalem, Israel) following the approval of the Institutional Ethical Committee for Care and Use of Animals. HAD1 hESC colonies were dissociated by trypsin digestion and 1.3×10^6 of passage 19 or 8×10^6 of passage 21 hESCs were injected into the rear leg muscle of 4–5-week-old mice. For the other cell lines, clusters of ~200 hESCs (HAD2, passages 14, 18 and 29; HAD3, passages 11 and 14; HAD5, passages 9 and 12) were mechanically removed from the feeders and injected under the testes capsule of 6-week-old NOD SCID mice (10–15 clumps per testis). After 6–14 weeks, the resulting tumors were removed, fixed in neutral buffered 4% formalin, embedded in paraffin and examined histologically after hematoxylin and eosin staining.

Results

Derivation of hESC lines from ICMs isolated by laser dissection

Human embryos that were diagnosed as genetically abnormal were recruited to the study from our PGD program between October 2005 and March 2006. Thirteen embryos developed to the blastocyst stage of which three were diagnosed as carriers of translocations and ten as carriers of monogenic

Table I. Characteristics of blastocysts and the outcome of laser-assisted hatching and ICM isolation.

Embryo no.	Development and morphological score*	Blastocyst age (days)	Plated on feeders as:	Blastocyst collapsed during:	hESC line
1	3AA	5	ICM		
2	5BA	7	Whole blastocyst	Assisted hatching	
3	5BA	7	Whole blastocyst	Assisted hatching	
4	5AA	6	ICM		HAD1
5	5AA	5	Whole blastocyst	Assisted hatching	
6	5AA	5	ICM		
7	5AA	5	ICM		HAD2
8	4AB	5	Whole blastocyst	Assisted hatching	
9	5AA	6	ICM		HAD3
10	5AA	5	ICM		
11	5AB	6	ICM		
12	6AA	6	ICM		
13	6BA	6	Whole blastocyst	ICM dissection	HAD5

*3, full blastocyst; 4, expanded blastocyst; 5, hatching blastocyst; 6, hatched blastocyst. ICM grading (first letter) A, tightly packed, many cells; B, loosely grouped, several cells; C, very few cells. Trophectoderm grading (second letter) A, many cells forming a cohesive epithelium; B, few cells forming a loose epithelium; C, very few cells.

diseases. Details regarding the age of the blastocysts and their developmental and morphological score at the time of ICM isolation are summarized in Table I.

For ICM isolation, the first step was to assist the hatching of unhatched or partially hatched blastocysts. By using micromanipulation techniques, hatching of the blastocysts was assisted by their gentle pulling through the whole in the zona pellucida that was made for blastomere biopsy during PGD (Fig. 1B).

The hatched blastocysts (Fig. 1C) were secured to the holding pipette, and the ICM was detached by laser pulses, perpendicular to the axis of the pipette (Fig. 1D–F).

Assisted hatching by micromanipulation was performed in eleven of the PGD blastocysts while two blastocysts were fully hatched at the time of ICM isolation. Four of the 11 blastocysts collapsed during the assisted hatching procedure and therefore were plated whole on human feeders (Table I).

Laser dissection of the ICM was attempted in nine PGD blastocysts. The ICM was successfully isolated in eight of these blastocysts. The reason for unsuccessful isolation in one blastocyst was its collapse during attempts to draw the ICM into the biopsy pipette. This blastocyst was plated whole on feeders (Table I).

The eight isolated ICMs and five whole blastocysts were plated on human foreskin feeders in hESC medium. Within 5–8 days, groups of small, tightly packed cells proliferated from the outgrowths of the ICMs and whole blastocysts (Fig. 1G). They were mechanically dissected from the outgrowth of differentiated cells and, following replating, gave rise to flat colonies of cells with the morphological appearance of hESCs (Fig. 2A). Three cell lines (HAD1–3) were established from the eight laser-dissected ICMs and an additional cell line (HAD5) was derived from the five whole blastocysts that were plated. The cell lines have been propagated in culture for prolonged periods (HAD1, HAD2, HAD3 and HAD5 for 41, 29, 43 and 40 passages, respectively) Early passaged cells from all cell lines were cryopreserved by both vitrification (Reubinoff *et al*., 2001) and conventional slow-cooling methods, and all cell lines were successfully recovered from cryopreservation.

Characterization of the hESC lines

The colonies of all cell lines displayed the typical characteristics of hESC colonies (Fig. 2A). They were compact with distinct borders and included cells with high nucleus to cytoplasm ratio and prominent nucleoli (Thomson *et al*., 1998; Reubinoff *et al*., 2000).

The cells of all four cell lines contained alkaline phosphatase activity (Fig. 2B). Indirect immunofluorescence staining showed that they were immunoreactive with anti-Oct-4 (Fig. 2C). Immunophenotyping of intact colonies demonstrated that the cells within them expressed Tra-1-60, Tra-1-81, SSEA-3 and SSEA-4 (Fig. 2E–H). All four cell lines showed normal diploid karyotype, HAD1 at passages 19, 28 and 37, HAD2, HAD3 and HAD5 at passages 24, 42 and 17, respectively (Fig. 2I). HAD1, HAD2 and HAD5 were derived from female embryos and HAD3 from a male blastocyst.

The pluripotent potential of the four cell lines was demonstrated by their capability to differentiate into progeny representing the three embryonic germ layers both *in vitro* and *in vivo*. Differentiation *in vitro* was either spontaneous into multiple lineages within EBs or controlled into neural precursors under specific defined culture conditions that blocked differentiation into lineages other than the neural one. (Itsykson *et al*., 2005). Indirect immunofluorescence studies of the differentiating outgrowths from plated EBs showed cells expressing muscle desmin (mesoderm) and sox-17 (endoderm) (Fig. 3A). Cells that were emanating from plated differentiating neural spheres were immunoreactive with anti-β-tubulin III antibody (neuronal marker; ectoderm; Fig. 3A).

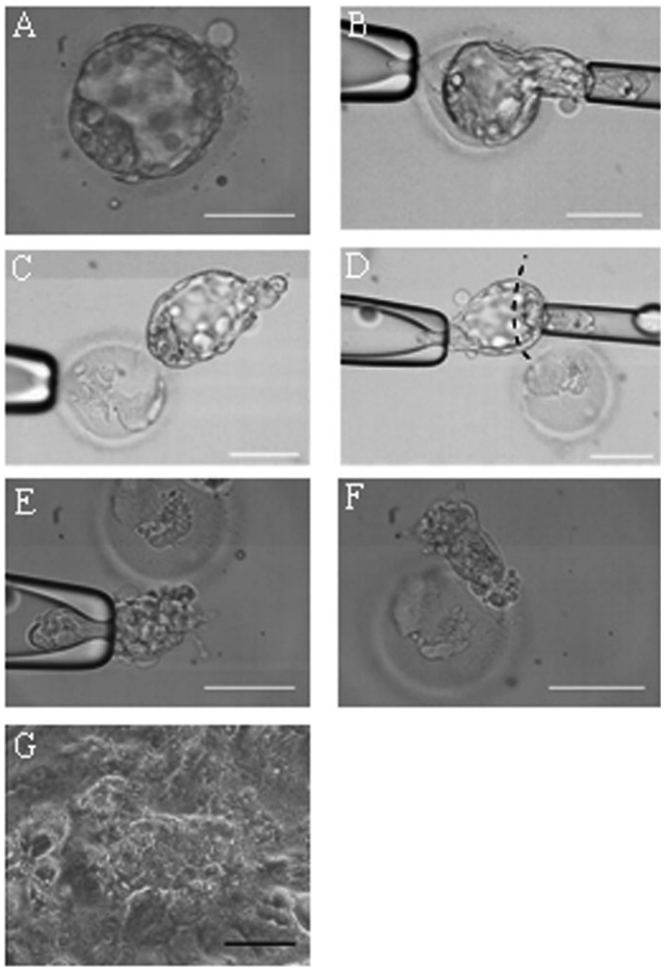

Figure 1: Laser-assisted dissection of the ICM
Nomarsky modulation optics images showing the isolation of the ICM of the hESC line HAD3: the blastocyst that was the ICM donor (**A**); assisted hatching of the blastocyst by micromanipulation through the laser-drilled orifice in the zona pellucida (**B**); the hatched blastocyst (**C**); the blastocyst during laser-assisted ICM isolation; laser pulses are aimed along the dashed line (**D**); the majority of TE cells secured to the holding pipette immediately after dissection and isolation of the ICM (**E**); the isolated ICM with remnants TE cells (**F**). Phase-contrast image of the outgrowth 4 days after plating of the ICM. Note the tightly packed cells within the surrounding differentiated cells, which following mechanical isolation and further propagation gave rise to HAD3 (**G**). Scale bars are 10 μm

When the cells were inoculated into the rear muscle (HAD1) or beneath the testis capsule (HAD2, HAD3 and HAD5) of SCID mice, lesions developed from all cell lines. Histological examination of the lesions 6–14 weeks after inoculation revealed that they contained cystic and solid areas of teratomas with differentiated tissues of the three germ layers (Fig. 3B).

Genetic analysis of the hESC lines

The cell lines were derived from embryos that were diagnosed as carriers of monogenic disorders. PGD was carried out on ideally two, or one, blastomeres that were biopsied at Day 3 of embryo development. F-PCR was used to analyse allele-specific mutations (when applicable) and linked polymorphic markers as previously described by Harper *et al*. (2002).

HAD1 hESC line was derived from a blastocyst donated by a couple in which the wife was affected with DM1 [120 $(CTG)_n$ repeat]. Linkage analysis showed that the embryo, as well as the HAD1 hESCs that were derived from it, received the expansion mutation. An expansion mutation of at least 150 repeats was confirmed in the hESCs using TP PCR (data not shown) (Warner *et al*., 1996).

HAD2 cell line was derived from an embryo that was donated by a couple carrying the 405+1G>A (husband) and the 2751+1insT (wife) CF mutations. The embryo was diagnosed as carrying the 405+1G>A mutation, but the analysis of the second CFTR allele was inconclusive. The couple decided to avoid further use of the embryo for fertility purposes and donated the embryo for stem cell derivation. Allele specific and lineage analysis by PCR showed that the HAD2 hESCs were carriers of the 405+1G>A mutation.

The embryo that gave rise to HAD3 was donated by parents of a hemophilia A-affected child. The familial mutation in this family was unknown, and therefore we used linkage analysis of polymorphic markers from the affected child and parents to diagnose that both the donor embryo and the HAD3 hESCs derived from it were carriers of the mutation. Both karyotype and PCR analysis showed that the HAD3 hESCs were of male gender.

HAD5 was derived from an embryo donated by a couple in which the wife was a carrier of fragile X permutation [with 80 repeats of $(CGG)_n$]. PCR analysis of the embryo and the HAD5 hESCs derived from it showed a female gender and the inheritance of the maternal X chromosome that was associated with the expansion mutation. Southern analysis of HAD5 hESCs further showed 300 $(CGG)_n$ repeats (data not shown).

Discussion

Our results demonstrate the use of a non-contact laser for the dissection of ICMs from the TE cells of PGD genetically abnormal human embryos and the establishment of hESC lines from these ICMs. Thus, laser dissection may serve as an alternative to other methods for ICM isolation and may be particularly valuable for deriving hESC lines in xeno-free conditions for potential future clinical applications.

Laser is commonly used in assisted reproductive technologies for a variety of applications, including assisted hatching (Obruca *et al*., 1994) as well as polar body, blastomere and TE biopsy (Veiga *et al*., 1997). Its utilization to assist in ICSI was also reported (Rienzi *et al*., 2001). The additional application of isolating the ICM, reported here, is technically simple and avoids the introduction of animal-derived reagents which are customarily used in immunosurgery (Thomson *et al*., 1998; Reubinoff *et al*., 2000; Cowan *et al*., 2004).

To facilitate isolation of the ICM, the blastocyst must first be extruded from the zona pellucida envelope. Dissolution of the zona pellucida may be accomplished by digestion with pronase; however, pronase is derived from bacteria and therefore is not optimal for the development of hESCs for future clinical applications. Chemical dissolution with acid tyrode is an alternative strategy (Ellerstrom *et al*., 2006) though it transiently acidifies the culture medium that may have an adverse

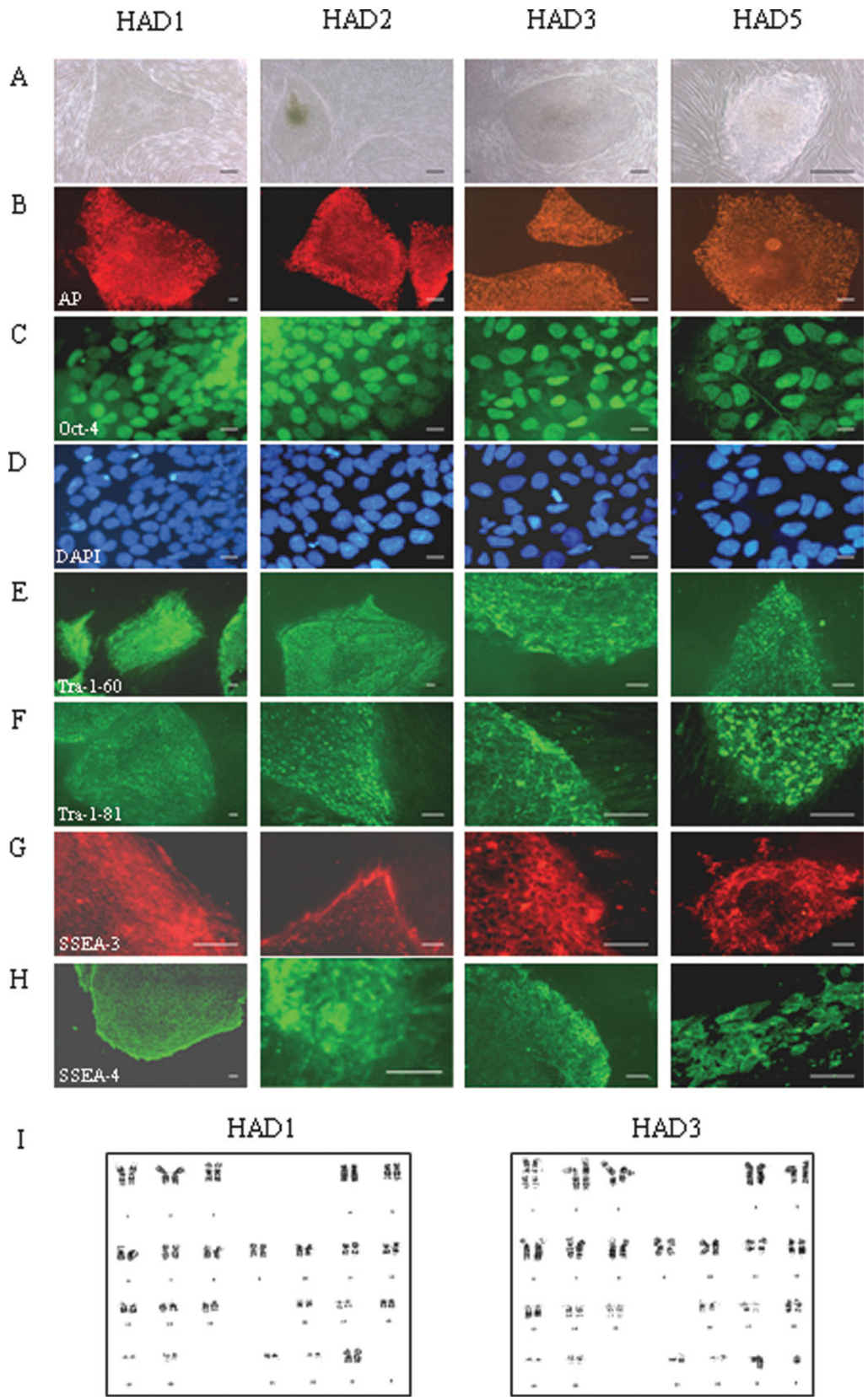

Figure 2: Characterization of the hESCs derived from laser-dissected ICMs and whole blastocysts
The hESCs that were derived from the PGD embryos formed colonies with typical morphology when cultured on foreskin feeder layer (**A**; phase-contrast images). The hESCs colonies expressed alkaline phosphatase (**B**; AP, fluorescence images). Indirect immunofluorescence staining showed that the hESCs were immunoreactive with anti-Oct-4 (**C**; nuclear 4′,6-diamidino-2-phenylindole counter staining, **D**). The undifferentiated hESC colonies also expressed Tra-1-60 (**E**), Tra-1-81 (**F**), SSEA-3 (**G**) and SSEA-4 (**H**). The hESCs had normal karyotype. A representative chromosome spread of HAD1 (46, XX) and HAD3 (46, XY) is presented in (**I**). Scale bars: A, B 200 μm; C, D 20 μm; E–H 100 μm

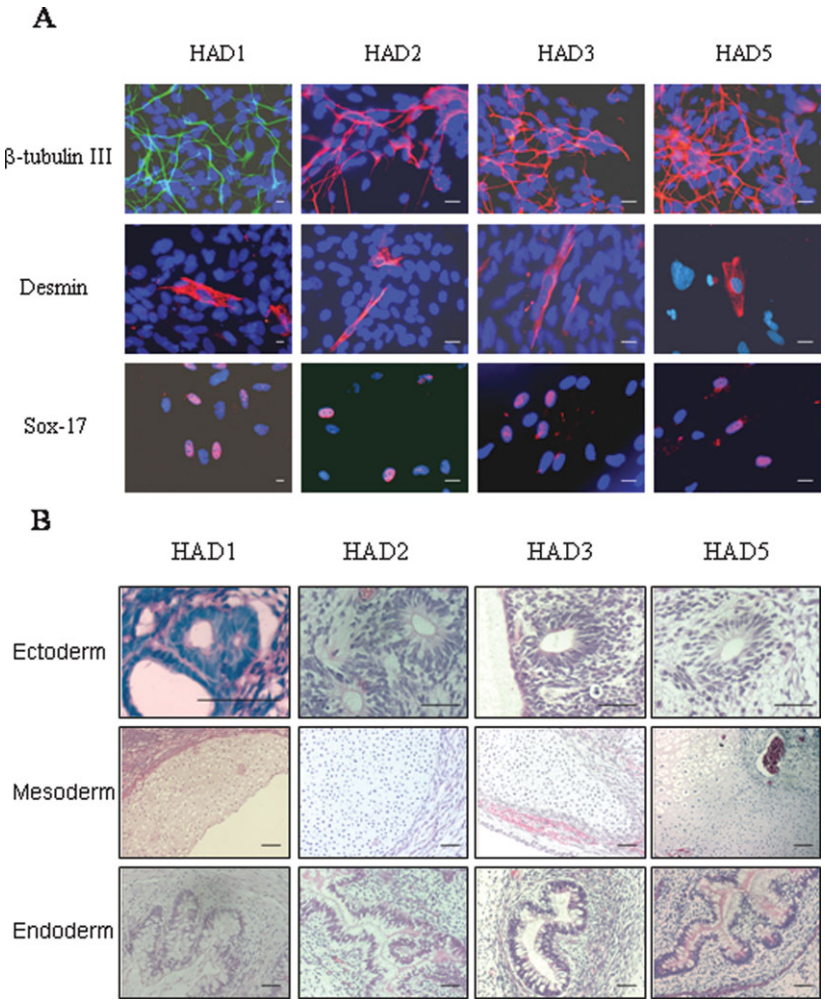

Figure 3: *In vitro* and *in vivo* differentiation into cells representing the three embryonic germ layers
(**A**) Differentiation *in vitro* was either spontaneous within Ebs or controlled into neural precursor spheres. After 3 weeks of differentiation by either method, the cells were plated for further 2–7 days of differentiation. Immunofluorescence staining showed that the differentiated cells from each of the lines expressed β-tubulin III, muscle desmin and sox-17, representing differentiation into ectoderm, mesoderm and endoderm, respectively. Scale bar: 20 μm. (**B**) Teratoma tumors developed 6–14 weeks after inoculation of hESCs into the rear leg muscle (HAD1) or under the testicular capsule (HAD2, HAD3 and HAD5) of NOD SCID mice. Hematoxylin–eosin stained histological sections of the tumors show neural rosettes, cartilage and structures with columnar epithelium that includes goblet cells representing differentiation into ectoderm, mesoderm and endoderm. Scale bar: 50 μm

effect on the blastocyst (Joris *et al*., 2003). Therefore, in this study, we have utilized the orifice in the zona pellucida that was created by laser at the cleavage stage for blastomere biopsy, to extrude the blastocysts by micromanipulation. It should be noted that in two embryos this procedure was not required since the orifice assisted spontaneous complete hatching. A similar approach may be applied to embryos that are not undergoing PGD. In these embryos, laser drilling of the zona pellucida should probably also be done at the cleavage stage rather than the blastocyst stage where the perivitelline space is minimal, rendering the TE cells more susceptible to heat damage with consequent blastocelle collapse.

During attempts to assist hatching by micromanipulation, collapse occurred in four out of eleven blastocysts. It seems that the frequency of blastocyst collapse decreased with experience. Identification and isolation of the ICM was impossible in collapsed blastocysts and therefore these blastocysts were plated whole on feeders. An alternative approach is to further culture the collapsed blastocysts to allow their re-expansion and then to dissect the ICM. Re-expansion of most collapsed blastocysts after TE biopsy was reported with further culture (McArthur *et al*., 2005).

In more recent studies than those described here, we used this approach for successful isolation of ICMs after the re-expansion of collapsed blastocysts (not shown).

The efficiency of derivation of hESCs from laser-assisted isolated ICMs was three lines from eight ICMs (37.5%). This efficiency is similar to the efficiencies reported after isolation of the ICM by immunosurgery (18–40%) (Thomson *et al*., 1998; Cowan *et al*., 2004; Ludwig *et al*., 2006; Mateizel *et al*., 2006), though prospective large randomized controlled studies are required to compare the efficacy of deriving hESC lines after isolation of the ICMs by various approaches.

Plating whole blastocysts on feeders was used in this study when the blastocysts collapsed during micromanipulation, as an alternative to isolation and plating of the ICMs. We

derived one hESC line after the plating of 5 whole collapsed blastocysts, confirming the reports of Heins *et al*. (2004) that hESC lines may be derived after plating of whole blastocysts. At present, it is unclear whether plating whole blastocysts on feeders is as efficient as plating isolated ICMs for the derivation of hESCs. Further studies will determine whether hESCs are more readily derived from isolated ICMs in comparison to plated intact blastocysts.

In this manuscript, we report the derivation of four hESC lines affected by mutations of DM1, hemophilia A, fragile X and CF (carrier). The derivation of hESC lines from PGD embryos carrying a variety of known genetic mutations is of utmost importance. These cell lines may serve as an invaluable *in vitro* tool for understanding the mechanisms of genetic disorders and developing new therapeutic approaches for the correction of these disorders, especially for diseases that are not mimicked by animal models. An international registry and possibly a repository of all hESC lines derived from PGD embryos worldwide should be established to promote their availability to the research community (Verlinsky *et al*., 2006).

In conclusion, we show the use of laser dissection for the isolation of ICMs from human abnormal PGD embryos. Laser-assisted isolation of ICMs circumvents the need for animal-derived reagents that are used for immunosurgery and therefore may be useful for the derivation of new xeno-free hESC lines that will be suitable for future clinical transplantation therapy.

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