Article

Establishment and characterization of new human embryonic stem cell lines

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Abstract

Human embryonic stem cells (hESC), with their ability to differentiate into all cell types in the human body, are likely to play a very important therapeutic role in a variety of neurodegenerative and life-threatening disorders in the near future. Although more than 120 different human embryonic stem cell lines have been reported worldwide, only a handful are currently available for researchers, which limits the number of studies that can be performed. This study reports the isolation, establishment and characterization of new human embryonic stem cell lines, as well as their differentiation potential into variety of somatic cell types. Blastocyst-stage embryos donated for research after assisted reproductive techniques were used for embryonic stem cell isolation. A total of 31 blastocysts were processed either for immunosurgery or direct culture methods for inner cell mass isolation. A total of nine primary stem cell colonies were isolated and of these, seven cell lines were further expanded and passaged. Established lines were characterized by their cellular and colony morphology, karyotypes and immunocytochemical properties. They were also successfully cryopreserved/thawed and showed similar growth and cellular properties upon thawing. When induced to differentiate *in vitro*, these cells formed a variety of somatic cell lineages including cells of endoderm, ectoderm and mesoderm origin. There is now an exponentially growing interest in stem cell biology as well as its therapeutic applications for life-threatening human diseases. However, limited availability of stem cell lines as well as financial or ethical limitations restrict the number of research projects. The establishment of new hESC lines may create additional potential sources for further worldwide and nationwide research on stem cells.

Keywords: *blastocyst culture, cryopreservation, embryonic stem cells, immunosurgery, in-vitro differentiation*

Introduction

Since their first establishment in 1998, several studies have reported the derivation and characterization of different human embryonic stem cells (Thomson *et al*., 1998; Reubinoff *et al*., 2000; Lanzendorf *et al*., 2001; Park *et al*., 2003; Heins *et al*., 2004; Pickering *et al*., 2004). Although more than 120 human embryonic stem cell lines exist at present, only a handful meet the criteria defined by the National Institutes of Health (NIH). Therefore, most of the research and findings on embryonic stem cells so far comes from reports utilizing only a couple of embryonic stem cell lines established by these groups.

However, besides the ethical and technical constraints on isolation and clinical use, immune rejection is another major issue to be resolved. Although it has recently been shown that it is possible to isolate human embryonic stem cells via 'therapeutic cloning', extremely low efficiency as well as moral acceptability of this technique makes it inapplicable in clinical cases for the time being (Hwang *et al*., 2004). Another approach proposed is to establish a 'stem cell bank' which contains hundreds of thousands immunophenotyped stem cell lines in order to match the recipient's HLA profile for immunocompetency (Trounson, 2002).

From this perspective, isolation and characterization of novel human embryonic stem cell lines is extremely important not only for expansion of the number of studies, but also for

increasing understanding of stem cell biology, which can lead to establishment of alternative cell therapy protocols. Besides other properties, embryonic stem cells are likely to prove a very valuable source for research involving mainly embryology, oncology, toxicology and pharmacology (Edwards, 2004).

This study reports the isolation and characterization of seven new human embryonic stem cell lines. These cell lines were also evaluated for their in-vitro differentiation properties into a variety of somatic cell types.

Materials and methods

The study was performed between January 2003 and May 2004 in Istanbul Memorial Hospital ART and Reproductive Genetics Centre. Thirty-one blastocyst-stage human embryos donated for research after assisted reproduction treatment were used. The study was approved by the IRB of Istanbul Memorial Hospital and informed consent was obtained from each donor couple.

Blastocyst-stage embryos included in the study were anonymously obtained from the embryology laboratory. Before they were processed for inner cell mass isolation or direct culture, they were morphologically evaluated according to scoring criteria reported elsewhere (Gardner *et al*., 2000).

Feeder cell preparation

Mouse embryonic fibroblast (MEF) cells isolated from inbred 12- to 14-day pregnant BALb/c mice were used as feeder cells. In all manipulations with MEF cells, media containing high glucose DMEM, 10% FBS (Gibco BRL; Invitrogen, Gaithersburg, MD, USA), 2 mmol/l L-glutamine (Gibco BRL), penicillin (50 IU/ml)/streptomycin (50 µg/ml; Gibco BRL) and mercaptoethanol (0.1 mmol/l; Sigma, Poole, Dorset, UK) were used. Cells between passages 2 and 6 were used for stem cell culture. Mitotic inactivation was performed by exposing the cells to mitomycin-C (10 µg/ml; Sigma) containing media for 3 h. Inactivated cells were trypsinized, seeded into culture plates covered with 0.1% gelatine (Sigma) and used as feeders after 48 h of incubation.

Isolation and preparation of human foreskin cells were as previously reported (Hovatta *et al*., 2003)

Inner cell mass isolation

It is generally agreed that the embryonic stage at which the immunosurgery procedure can be applied is mainly determined by the degree of trophectoderm expansion with intact and apparent inner cell mass, which usually occurs between days 5 and 7 after insemination. Moreover, culture of spare human blastocyst-stage embryos for more than 7 days generally leads to degeneration in routine human IVF culture media. Therefore, on day 5 or 6 after fertilization, all blastocysts that were selected for embryonic stem cell isolation were processed for zona pellucida removal by short-term exposure (2–5 min) to embryo culture media containing pronase (Sigma).

Immunosurgery was performed according to a previously published protocol (Solter and Knowles, 1975). Antibody against human albumin (Sigma) and guinea pig complement (Accurate Chemical, Westbury, NY, USA) were used for lysing and removing trophoblastic cells. Further cell and debris removal was performed by serial pipetting with decreasing inner diameters. Intact inner cell mass clumps were then transferred onto MEF culture containing embryonic stem cell culture media and cultured for 7–10 days.

Alternatively, after the zona removal, direct culture was performed by transferring zona-free human blastocysts onto previously prepared feeder cells in the presence of complete stem cell media. Culture media was changed every day and cellular and colony morphology was recorded until the appearance of the first stem-cell like colonies, which was around 7 days after initial plating.

Embryonic stem cell culture

Stem cell culture media included Ko-DMEM (Gibco BRL), 15% FBS (Hyclone, South Logan, UT, USA), 2 mmol/l L-glutamine (Gibco BRL), penicillin (50 IU/ml)/streptomycin (50 µg/ml; Gibco BRL), non-essential amino acids (×1; NEAA, Sigma), β-mercaptoethanol (0.1 mmol/l; Sigma), insulin–transferrin–-selenium complex (×1; ITS; Gibco BRL) and human recombinant leukaemia inhibitory factor (LIF) (12 ng/ml; Chemicon, Temecula, CA, USA). After an initial eight passages, LIF was omitted from culture media.

Karyotyping and immunocytochemistry

Stem cell colonies were cultured in media containing 0.1 µg/ml colcemide (Biological Industries Ltd., Kibbutz Beit Haemek, Israel) for 3 h at 37°C, 6% CO_2. Colonies were then mechanically isolated from MEF feeders and transferred into 15 ml conical Falcon tubes containing cell culture media. After centrifugation at 145 *g* for 10 min, the pellet was resuspended with hypotonic medium (0.075 mol/l KCl). Cells were then incubated for 17 min at 37°C, fixed with methanol/acetic acid (3:1) and processed for G banding.

For immunocytochemistry, the cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; Gibco BRL) for at least 15 min. Antibodies raised against surface antigens SSEA-4, TRA-1–60, TRA-1–81 and alkaline phosphatase substrates was obtained commercially as a stem cell characterization kit (Chemicon). Analysis was performed according to manufacturer's instructions by using FITC-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and epifluorescence microscope (BX50; Olympus, Tokyo, Japan). Other antibodies such as OCT-4, nestin, microtubule associated protein (MAP) 2A and B and cardiac-specific troponin I used in this study were also purchased from Chemicon.

Freezing/thawing procedures

Vitrification was performed according to the protocols published previously with minor modifications (Reubinoff *et al*., 2001; Vanderzwalmen *et al*., 2003). After mechanical cutting from culture plates, colonies were first transferred into medium containing HEPES and equilibrated for 1 min. They

were then cultured in solution I (containing 10% DMSO and 10% ethylene glycol) for 1 min and transferred to solution II, 20% DMSO, 20% ethylene glycol and 0.3 mol/l sucrose, for a maximum of 30 s. At the end of the incubation period, colonies were collected with the mouth pipette and transferred to the tip of the cryostraw.

Warming was performed by sequentially incubating the colonies in media containing 0.2 mol/l and 0.1 mol/l sucrose for 1 min each and media containing HEPES for 5 min. Colonies were then transferred onto feeder cells culturing in embryonic stem cell media.

Results

A total of 31 blastocysts donated for research were used. All blastocysts were examined in detail under inverted microscope for their degree of expansion and cellular morphology. During the first phase of the study, 20 of the blastocysts with variable quality of inner cell mass were donated for research and selected for human embryonic stem cell isolation. Fifteen were found to be suitable for immunosurgery (**Table 1**). Twelve intact inner cell mass clumps were obtained and cultured on mitomycin-C-inactivated mouse embryonic fibroblast cells (**Figure 1**).

During the initial culture period of 7–10 days on MEF, primary stem cell-like colonies were observed in four ICM clumps (4/12; 33.3%) They were named as NS-1, NS-2, NS-3 and NS-4 and mechanically passaged onto a new feeder environment every 5–7 days. After passage 3, NS-1 and NS-2 cells were found to be spontaneously differentiated before they were effectively cryopreserved, and hence were lost in culture. The other two cell lines (NS-3 and NS-4) were successfully vitrified/warmed several times and have been continuously growing on feeder cells for more than 40 passages (**Figure 2A–C**). Similar growth rates were also observed for both cell lines as assessed by colony growth at certain time points during culture (**Figure 3**). These parameters were found to be similar after vitrification and subsequent warming experiments, which resulted in ≥70% survival rate after warming for all cell lines (data not shown).

During the second phase of the study, direct culture of zona-free blastocysts on feeder cells was performed for 11 blastocysts. Five of these embryos were again obtained from routine assisted reproduction treatment cycles as leftover donated material, not eligible for freezing. After 7–10 days of initial culture on feeder cells in the presence of stem cell culture media, stem cell-like colony growth was observed in one plate. These cells were isolated from surrounding differentiated or trophoblastic cells by careful mechanical isolation with finely drawn glass Pasteur pipettes and expanded by subsequent passaging (thereafter named as MINE).

Another six blastocysts were donated by a couple undergoing preimplantation genetic diagnosis (PGD) for HLA typing. After analysing the embryos for possible HLA matching, those that were not HLA compatible with the affected child were donated for research. After manipulation of these blastocysts for direct culture and transferring them onto the feeder environment, four out of six blastocysts successfully generated primary stem cell colonies (66.7%). These cell lines were named NS-5, NS-6, NS-7 and NS-8. They were expanded, vitrified/warmed with the other cell lines mentioned before (data not shown).

Unlike the mouse counterpart, which is required for the feeder-free growth of murine embryonic stem cells, human embryonic stem cells spontaneously differentiate under a feeder-free environment even in the presence of LIF. There is also no documented positive (or negative) effect of inclusion human LIF in the derivation or the initial culture phase. However, several other groups have also reported the inclusion of this compound in the establishment phase or in the routine culture periods of human embryonic stem cells (Klimanskaya and McMahon, 2004). For this reason, LIF was added in the initial derivation and culture phase, but removed in the later culture stages (more than eight passages). No negative effect of LIF removal was observed on the cell/colony morphology and growth characteristics of cultured human embryonic stem cells beyond this stage.

In order to characterize the established cell lines, a general approach that involves the overall analysis of cells according to cellular and colony morphology, immunocytochemical characteristics, such as expression of certain surface antigens (SSEA-4, TRA-1–60, TRA-1–81 and alkaline phosphatase) as well as karyotyping, was sought.

All the lines showed continuous proliferation with stable round-shaped colonies with distinct borders. Under higher magnification, cells with prominent nucleoli as well as high nucleus to cytoplasm ratio were evident, consistent with previous reports (**Figure 2A–C**). All seven established cell lines were also found to be similar to other previously reported human embryonic stem cells expressing common markers such as SSEA-4, TRA-1–60, TRA-1–81 and alkaline phosphatase (**Figure 2E–H**). All had normal karyotypes as shown after G-banding; a representative picture for NS-3 is shown in **Figure 2D**.

Human embryonic stem cells, when grown in suspension without feeder cells and LIF, spontaneously form three-dimensional clumps and differentiate into derivatives of all three germ layers. Therefore, the pluripotency of each individual cell line established in this study was tested by in-vitro embryoid body formation and subsequent culture on feeder-free environment. After being mechanically isolated from feeder cells, cell clumps were initially cultured in bacterial Petri dishes for 7–10 days. The resulting cystic embryoid bodies were transferred onto gelatine-coated plates and cultured for further cell growth and differentiation for 4 weeks. During this time, a variety of different cell types, including spontaneous beating areas, neuron-like cell aggregates, epithelial as well as endothelial-like cells, were observed in growing embryoid bodies (**Figure 4**). Differentiating cells in embryoid bodies showed nestin and MAP2A and 2B expression and the cardiac-specific nature of spontaneous beating cells was detected by immunocytochemical analysis using an antibody against cardiac troponin I (**Figure 5**).

All human embryonic stem cell lines isolated in this study were analysed for their ability to grow on human foreskin cells

Table 1. Morphological evaluation of blastocysts processed for immunosurgery. ESC = embryonic stem cell.

No.	*Developmental stage*	*Grade*	*Immunosurgery*	*ESC established*
1	Early blastocyst			
2	Expanded blastocyst	BC	+	
3	Expanded blastocyst	BC	+	
4	Hatching blastocyst	BB	+	
5	Expanded blastocyst	AB	+	+
6	Expanded blastocyst	BC	+	
7	Hatching blastocyst	BB	+	+
8	Hatching blastocyst	BC	+	
9	Expanded blastocyst	BB	+	+
10	Early blastocyst			
11	Blastocyst	BA	+	+
12	Expanded blastocyst	BC	+	
13	Expanded blastocyst	BC	+	
14	Expanded blastocyst	BC	a	
15	Blastocyst	BC	+	
16	Expanded blastocyst	BC	+	
17	Hatching blastocyst	BC	+	
18	Blastocyst	BB		
19	Hatching blastocyst	BC	+	
20	Early blastocyst			

[a]Collapsed in culture therefore was not processed for immunosurgery.

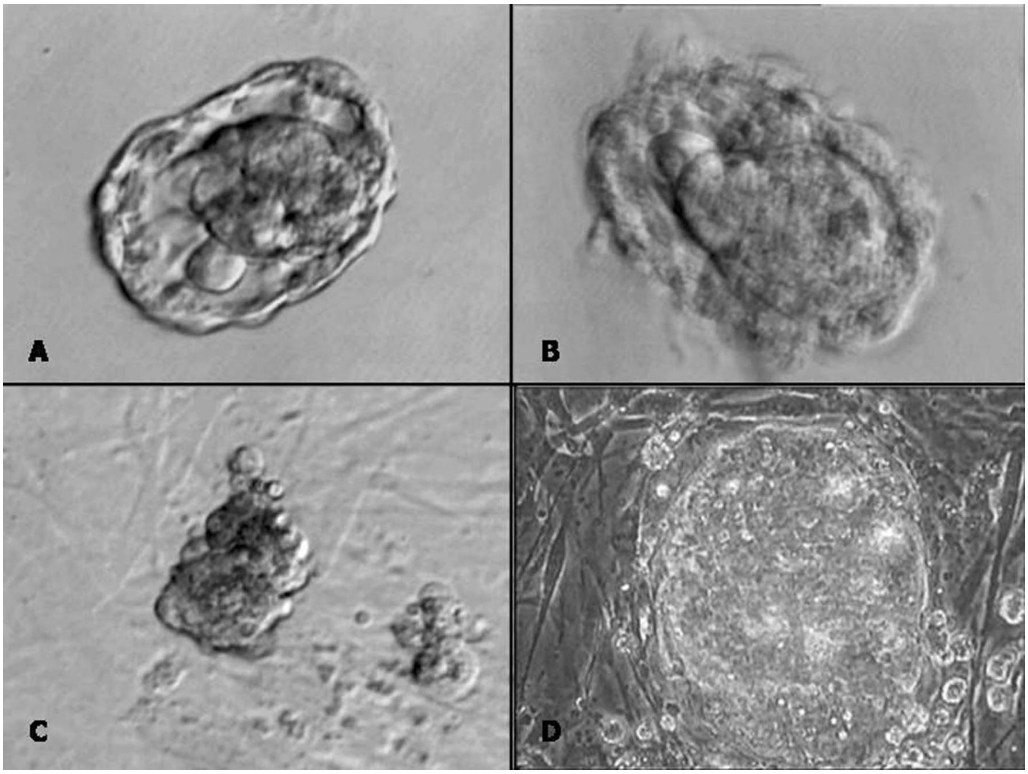

Figure 1. (**A**) A human blastocyst with an apparent inner cell mass (ICM). (**B**) Trophectoderm cells being lysed after guinea pig complement treatment. (**C**) An inner cell mass clump obtained after serial pipetting of the blastocyst in (**B**). (**D**) Primary stem cell colony obtained after cultivation of ICM clump on feeder cells. Original magnifications: A, B, C – ×400 (Hoffmann modulation); D – ×100.

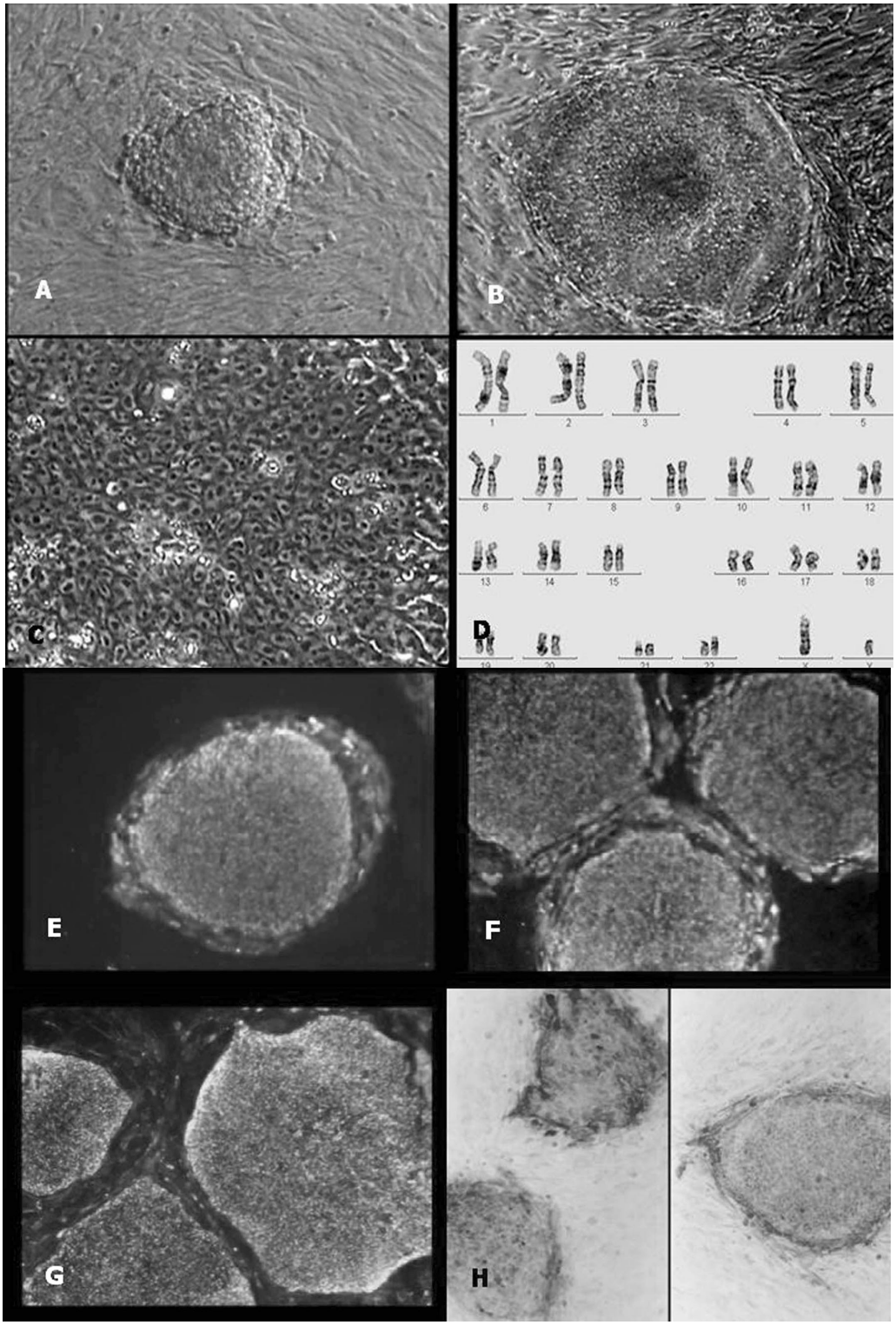

Figure 2. (**A**) NS-3 stem cell colony after first passage. (**B**) An embryonic stem cell colony after 6 days of culture on mouse embryonic fibroblasts. (**C**) A typical embryonic stem cell morphology (×200 magnification). (**D**) NS-3 cell line showing a normal human karyotype of 46,XY. (**E–G**) Immunocytochemistry results of NS-4 cell line against surface antigens stage-specific embryonic antigen-4, TRA-1–60 and TRA-1–81. (**H**) Alkaline phosphatase expression of NS-3 and NS-4 colonies. Original magnifications: A – ×40; B – ×40 (Hoffmann modulation); C – ×400; E, G, F, H – ×40.

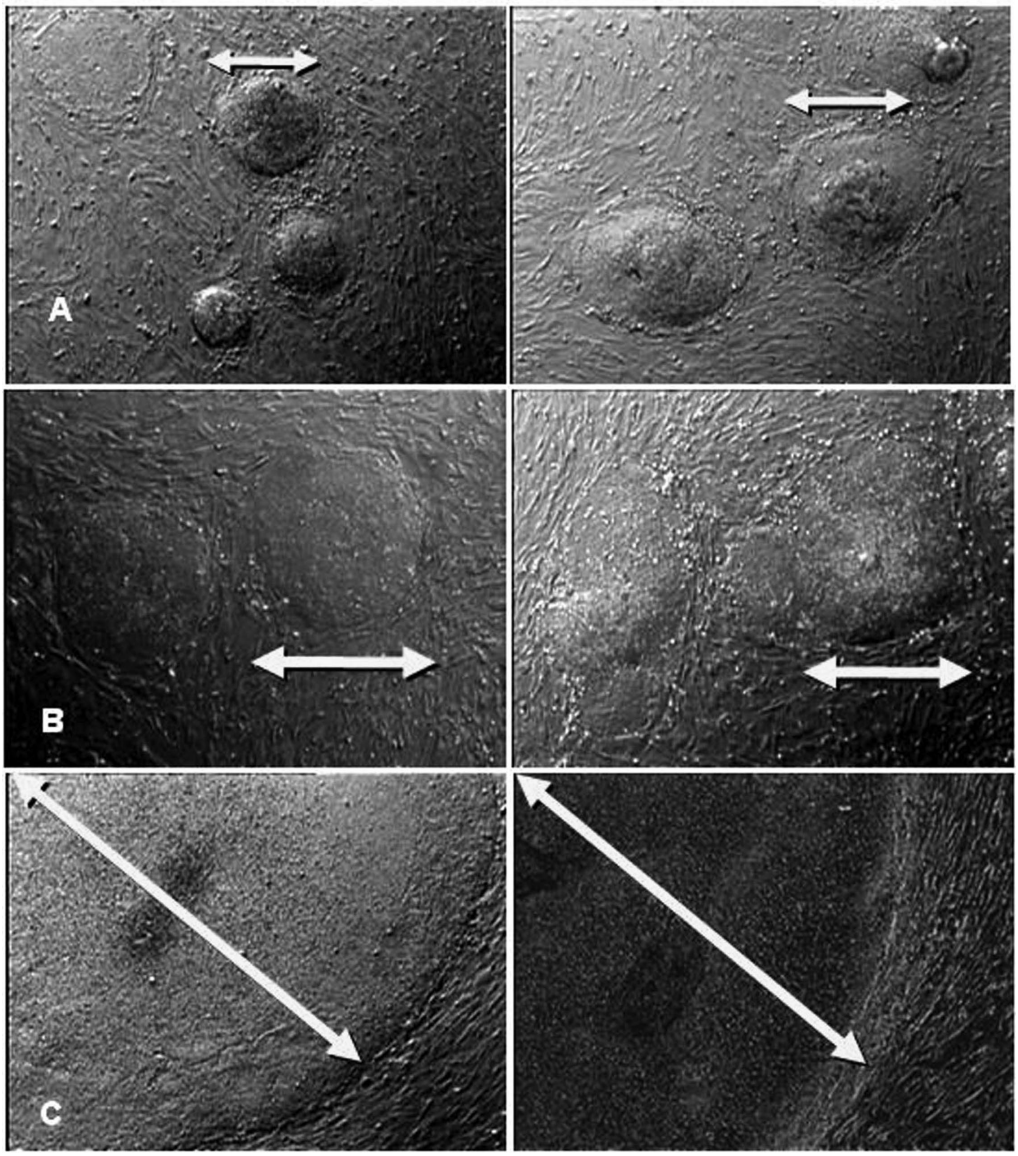

Figure 3. Colony growth of human embryonic stem cell lines NS-3 (left) and NS-4 (right) at (**A**) 24 h, (**B**) 72 h, and (**C**) 144 h after initial plating. Original magnification: ×40 (Hoffmann modulation). Arrows show the relative growth of similar-sized clumps at certain days of culture (day 1, day 3 and day 6) on the mouse embryonic fibroblast (MEF) layer. These arrows emphasize the similar growth characteristics of both lines. Since they were passaged mechanically, a routine cell counting protocol could not be applied to indicate the exact doubling time.

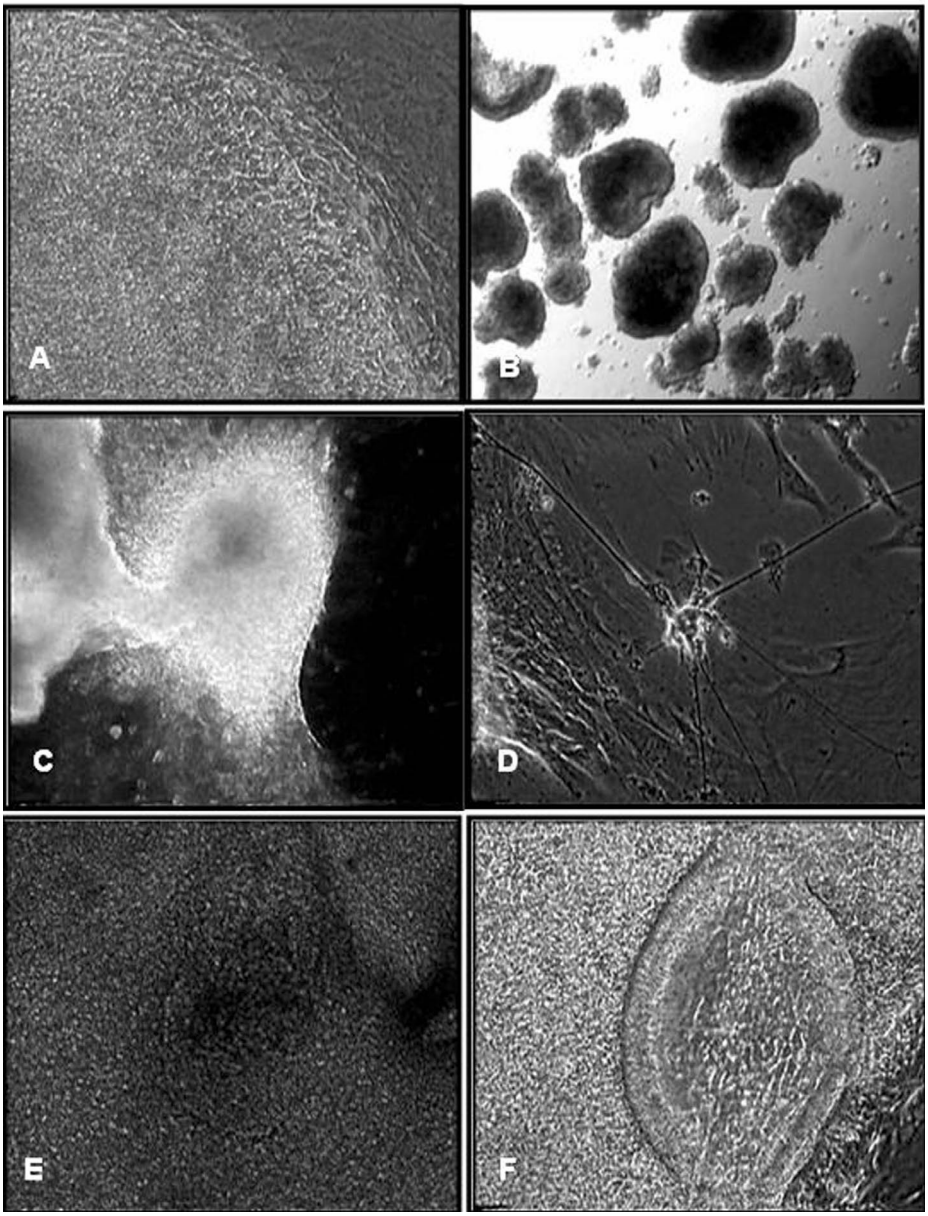

Figure 4. In-vitro differentiation of human embryonic stem cell lines. (**A**) Undifferentiated embryonic stem cell colony (NS-3). (**B**) Six-day-old embryoid bodies in suspension culture. (**C**) Attachment and growth of embryoid bodies on tissue culture plate. (**D**) Differentiation of human stem cells into neuron-like cells. (**E**) Areas showing spontaneous 'beating'. (**F**) Epithelial-like cells. Original magnifications: A, D – ×100; B – ×40 (Hoffmann modulation); C, E, F – ×400.

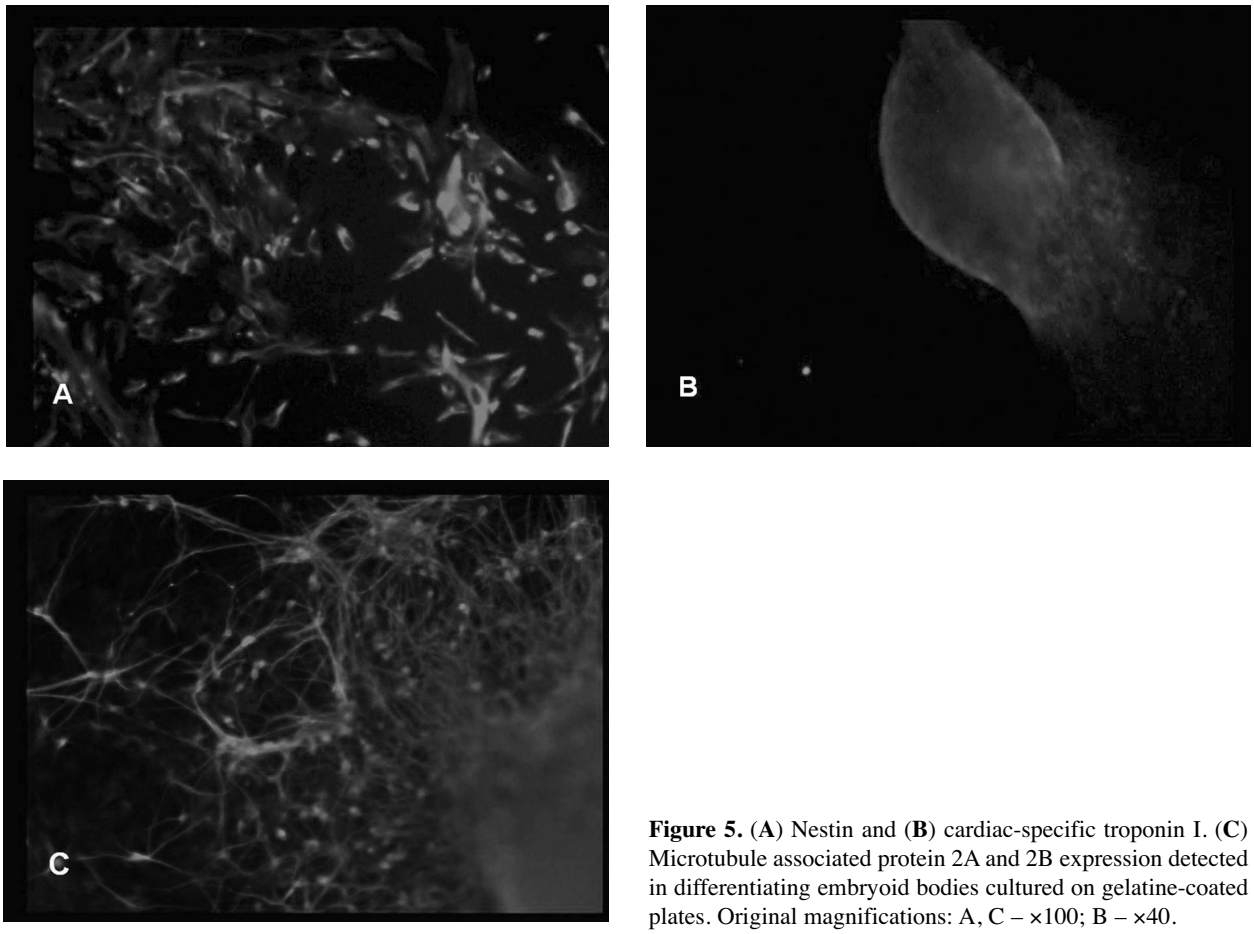

Figure 5. (**A**) Nestin and (**B**) cardiac-specific troponin I. (**C**) Microtubule associated protein 2A and 2B expression detected in differentiating embryoid bodies cultured on gelatine-coated plates. Original magnifications: A, C – ×100; B – ×40.

Table 2. Overall outcome of human embryonic stem cell isolation procedures.

	Blastocysts used (n)	*ESC isolated* (n)
Isolation technique		
Immunosurgery	15	4 (26.6)
Direct culture	11	5 (45.5)
Source of embryos		
Spare, not suitable for freezing	20	5 (25.0)
HLA incompatible	6	4 (66.6)
Total	26	9 (34.6)

Values in parentheses are percentages.

as feeder cells of human origin. After and initial culture on MEF (>15 passages), a subgroup of undifferentiated colonies were mechanically cut and transferred onto foreskin cells which were previously inactivated with mitomycin C. All the colonies showed attachment to foreskin feeders within 24 h and continued to grow on subsequent culture days. Except for a slight change in the colony morphology, which becomes polarized according to the direction of foreskin cells, other growth characteristics were observed to be similar for all cell lines (data not shown).

Discussion

Limited amounts of immunocompetent donor material for organ and tissue transplantation, together with the extremely high cost, make current therapeutic strategies very inefficient in providing a cure for many lethal diseases, including myocardial infarcts, neurodegenerative disorders such as Parkinson's or Alzheimer's disease as well as diabetes. Although adult and cord blood stem cells can provide an alternative source for cell therapy, similar limitations exist for these cells unless autologously used. Autologous use, on the other hand, although it minimizes the ethical as well as legal problems associated with the isolation and donor–recipient issues, may not produce enough cells for transplantation in many cases. Moreover, due to the lack of standard application as well as research-based protocols, clinical results obtained on limited number of cases, as well as results of in-vitro differentiation studies, remain largely unknown, and therefore need to be validated.

All of these debates highlight the prospective use of human embryonic stem cells as another potential and abundant source in both in-vitro and transplantation studies. Although the isolation and culture of these cells raises significant ethical and legal issues worldwide, their self-renewing nature as well as the ability to be indefinitely cultured *in vitro* without any signs of differentiation makes human embryonic stem cells a very important candidate source for cell-based therapy systems (Edwards, 2004). In that sense, having as many human embryonic stem cell lines as possible is likely to increase the speed of both in-vitro and in-vivo research studies, which can result in a potential therapeutic tool in the near future. In addition, although therapeutic cloning may in theory serve as an alternative approach for eliminating immune mismatch between donor and the recipient, the extremely low success rate, lack of technical and biological validation, as well as ethical issues, indicate that this approach is unlikely to provide an alternative in the near future.

In this study, by using both immunosurgery and direct culture, seven new human embryonic stem cell lines have been established out of 31 blastocyst stage embryos (22.6%). All the lines showed common properties that are similar to those previously reported pluripotent human embryonic stem cells.

Consistent with previous studies, the efficiency of successful derivation varied greatly, and was largely dependent on the quality of the blastocysts in the present study. Successful derivation was observed only from blastocysts with visible inner cell mass clumps, having A and B grade ICM (**Table 1**). On the other hand, six good quality blastocysts obtained from a PGD-HLA typing cycle resulted in four established embryonic stem cell lines, indicating that the quality of the starting material is in fact the most critical parameter (**Table 2**).

It has been suggested that relatively lower success rates observed in human embryonic stem cell isolation can also be due either to species-specific and intrinsic biological as well as functional properties or inefficient and suboptimal technology utilized in the derivation process. In the majority of studies, fresh or frozen–thawed spare human embryos have been used as a source material. Very few of the fresh spare embryos can survive and form blastocysts in extended culture, which results in lower rates of embryonic stem cell derivation compared with frozen–thawed counterparts (Sjögren *et al.*, 2004). In addition, most of the cell lines established so far have utilized immunosurgery. Since in this protocol, exposing the blastocysts to antibodies raised in animals is necessary, the isolated cells may not be suitable for therapeutic use in the future. Therefore, efficient alternative isolation methods need to be developed in order to use them in future transplantation studies.

Several groups have recently reported the establishment of new human embryonic stem cell lines via direct culture methods (Heins *et al.*, 2004; Sjögren *et al.*, 2004; Suss-Toby *et al.*, 2004). In this study, although the number of materials was limited, a tendency of improved success with direct culture was observed (26.6 versus 45.5%), consistent with the results of previous studies.

All of the cell lines established in this study were found to have normal karyotypes and to express high concentrations of surface antigens and alkaline phosphatase. The ability of established lines to generate differentiating cells comprising all three germ layers were examined *in vitro* by culturing the cells in suspension in the absence of feeder layers. The resulting cystic embryoid bodies were plated on gelatine-coated tissue culture plates for further differentiation during prolonged culture, as previously reported (Itskovitz-Eldor *et al.*, 2000). Embryoid bodies generated from individual lines showed numerous populations of cells with variable morphology, including spontaneously beating areas, neuron-like cellular bundles as well as other somatic cell types, indicating that they all carry the potential to form cellular types originated from all germ layers. Following recently varying results on cardiomyocyte differentiation capacities of different human embryonic stem cell lines, it is necessary to note that the percentage of embryoid bodies with spontaneous contractions in this study was around 8–10% and this rate was similar with different cell lines tested. These data will be published in a future paper.

Isolated human embryonic stem cell lines were also tested for their ability to grow on human foreskin fibroblast cells. Cells grown on this human-origin feeder system showed comparable colony morphology and growth characteristics with that of mouse embryonic fibroblasts, as reported previously by other groups (Richards *et al.*, 2002; Hovatta *et al.*, 2003). Moreover, recent reports also indicate other potential feeder sources of human origin such as endometrial stromal cells, marrow stromal cells, fetal muscle and skin cells, which are compatible with the human embryonic stem cell culture (Richards *et al.*, 2002; Amit *et al.*, 2003; Cheng *et al.*, 2003; Hovatta *et al.*,

2003; Lee *et al*., 2004). Other alternative culture methods such as feeder-free culture are of another area of interest in current research (Xu *et al*., 2001; Carpenter *et al*., 2003; Koivisto *et al*., 2004). Therefore, the authors of this article believe that finding derivation and maintenance systems for embryonic stem cells that are devoid of pathogenic animal material will greatly help scientists working on stem cells and transplantation in order to develop effective methods for cell therapy. A project on the isolation and detailed characterization of human embryonic stem cells on different human feeder cells is currently underway.

Human embryonic stem cell lines isolated in this study, as well as the cell lines in the previous reports, could be another potential source material in in-vitro studies involving basic molecular and stem cell biology. There is already growing interest for embryonic stem cells in toxicology and drug therapy. Moreover, the use of embryos after PGD, with their intrinsic genetic content as well as the disease profile, could be an extremely valuable source for such research on genetic diseases (Pickering *et al*., 2003). PGD combined with HLA typing has now become a therapeutic tool for certain diseases which can be cured using adult stem cell transplantation (Kuliev and Verlinsky, 2004). This study reports the successful isolation of human embryonic stem cells from four out of six embryos donated after PGD combined with HLA-typing cycle. Although the number of embryos was too limited to draw a conclusion, the relatively high quality of the embryos in these cycles compared with an infertile population (Kahraman *et al*., 2004) may indicate that this technique can provide an alternative to adult stem cells, being an unlimited stem cell source for affected individuals in the future.

So far as is known, this study is the first report on the derivation, characterization and long-term maintenance of human embryonic stem cell lines from donated human blastocyst-stage embryos in Turkey. In some countries, stem cell research involving human embryos is strictly regulated and under the control of several committees or institutions. On the one side, there are some countries in which research on human embryos is strictly prohibited, such as Germany, Norway and Italy. At the other extreme, not only the use of human embryos for research but also the therapeutic cloning for human stem cell research is allowed in the United Kingdom. Turkey is among several countries in which no specific regulations and guidelines have so far been defined by legal or governmental institutions for stem cell research. Therefore, studies such as the one reported in this article on stem cell research can create scientific as well as social interest and eventually help these countries to define appropriate guidelines and regulations.

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