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Derivation and Characterization of New hESC Lines from Supernumerary Embryos, Experience from Turkey

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1. Introduction

Human embryonic stem cell (hESC) lines, which are derived from inner cell mass (ICM) of supernumerary blastocysts-stage embryos, have well known unique properties; long-term self-renewing ability with maintenance of an undifferentiated state and pluripotent capacity to differentiate into all derivatives of three embryonic germ layers (Hoffman & Carpenter, 2005; Semb, 2005; Trounson, 2006). Since its first derivation and characterization by Thomson et al in 1998, these intrinsic properties have made hESC very popular worldwide and, thereby, many studies describing isolation and characterization of new hESC lines have been reported (Findikli et al., 2005; Simon et al., 2005; Thomson, 1998). HESCs have been considered very valuable and promising cell source for research involving mainly human embryogenesis, oncology, drug toxicology and developmental biology as well as for cell based regenerative therapies (Edwards, 2004).

Obviously, studies on hESCs mostly focus on their potential use for treatment of degenerative human diseases. However, due to the largely unknown characteristics of established lines and the use of animal based material in their cultures, most of the lines could not be suitable for prospective transplantation studies (Findikli et al., 2006; Rodriguez et al., 2006). Therefore, registration of existing hESC lines with their characteristics in stem cell banks would provide database of cell lines, cooperation and co-regulation for researchers.

In this chapter, it was aimed to report the methods to derive 18 hESC lines which were established and characterized until the declaration of prohibition on hESC research in Turkey by Health Ministry in 2005. Additionally, it was discussed the current legal situation of hESC research and perspectives to that issue in Turkey.

2. Material and methods for derivation and characterization of hESC lines

Derivation and characterization of all hESC lines were undertaken in Memorial Hospital ART &Reproductive Genetics Centre, R&D Laboratory, Istanbul, Turkey between January 2003 and September 2005. All donated supernumerary embryos were used after obtaining written informed consents from couples. All hESC lines were established only for research purpose rather than for any financial interest. This study was approved and controlled by the local ethic committee/Internal Review Board of Istanbul Memorial Hospital.

2.1 Source of supernumerary human embryos

Supernumerary human embryos used for derivation of hESC lines were obtained after in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) and preimplantation genetic diagnosis (PGD) cycles. Most of these embryos had poor quality and thereby, were considered insufficient for replacement or cryopreservation.

Preimplantation genetic diagnosis is applied to three groups of patients with variety of indications in our clinic. The first group of PGD includes patients who have a high risk of transmitting their single gene disorder to their offspring. After diagnosis, abnormal embryos were used to derive hESC lines with specific single gene mutation.

In the second group of PGD cycles, embryos belonging to couples with advanced maternal age, a history of recurrent miscarriages and repeated implantation failures are destined to chromosomal screening (Kahraman et al., 2000, 2006; Lavon et al., 2008; Munne et al., 2005; Verlinsky et al., 2005). Following PGD, chromosomally abnormal embryos were subjected to derivation of hESC lines having chromosomal aneuploidies.

In the third group, PGD is used for identification of embryos for human leukocyte antigen (HLA) matching to an affected older sibling who requires hematopoietic stem cell transplantation. Furthermore, the HLA matching can be combined with mutational analysis for genetic diseases in cases where the sibling is affected with this monogenic disorder and waiting for stem cell transplantation. Therefore, in those cases, embryos having mismatched HLA type or carrying genetic disorder were used for derivation of hESC lines.

2.2 Isolation and preparation of feeder cells

As feeder cells both mouse embryonic fibroblast (MEF) and human foreskin fibroblast (HFF) were used during isolation and long term culture of hESC lines. MEFs were isolated from embryos of the 12- to 14-day pregnant BALb/c mice (Conner, 2000). To isolate single cell suspension of MEF mouse embryos isolated from the sacrificed mice by cervical dislocation were dissociated into small pieces with scissor. Then dissociated tissues were trypsinized in 0.25% trypsin-EDTA (Gibco BRL; Invitrogen, Gaithersburg, MD, USA) for 15min to produce single cell suspension.

Human foreskin fibroblasts were isolated from circumcised tissues of 0-1 year old males. Cell isolation was performed as described previously (Hovatta et al., 2003; Richard et al., 2002). In a brief, following the isolation of dermis from the epidermis by scissor or razor blade, tissue was dissected into small pieces and then trypsinized in 0.05% trypsin-EDTA (Gibco BRL) for approximately 1 h to dissociate into single cells.

Both 25 cm^2 and 75 cm^2 culture flasks were used to culture HFF and MEF lines. These lines were grown in feeder cell culture medium, consisting of 85% high glucose DMEM (Gibco BRL), 10%FBS (Gibco BRL), 1% penicillin streptomycin-amphotericin (Biological Industries) and 2mM L-glutamine (Gibco BRL) at 37°C with 5% CO_2. Supportive medium was changed in every three days. MEF lines could be used in culture of hESC lines up to 6 passages, whereas HFFs had supportive potential up to 15 passages.

Mitotic inactivation of feeder cells was performed after exposing feeder cells to culture medium containing 10μg/ml mitomycin C (Sigma-Aldrich, Poole, Dorset, UK) for 2.5-3h. Inactivated cells were seeded on a 0.1% gelatin-coated at a concentration of 1.5×10^5 cells / ml. After 2 days incubation organ culture dishes were ready to use as feeder plates. Feeder plates could be used for the following 7 d.

2.3 Isolation and long-term culture of hESC lines

Blastocyst stage embryos, that were graded according to the Gardner's scoring criteria, were processed for hESC isolation by either immunosurgery or direct culture (Gardner et al., 2000; Findikli et al., 2005). Prior to the immunosurgery or direct culture, zona pellucida of embryo was removed by the short-term exposure to the 5IU/ml (final conc.) pronase (Sigma) containing embryo culture medium for up to 5 min. Immunosurgery was applied based on the previously published protocol (Solter and Knowles, 1975). After lysis of trophoblastic cells, the resulted intact inner cell mass clumps were placed on feeder cells and cultured until to observe the primary hESC colonies.

In the direct culture method, zona free blastocysts were directly placed on feeder cells and cultured until the appearance of outgrowth, which lasted about 6 to 8 days. Then compacted outgrowths including cells of hESC-like morphology were mechanically split into small clumps. The cell clumps were transferred on new feeder plates. The primary colonies were generally observed after about 5 to 7 days (Figure 1A-B).

HESC lines were cultured at 37 °C in 5% CO_2 in the complete stem cell medium (CSCM) with the composition of 85% Ko-DMEM (Gibco BRL), 15% FBS (Hyclone, South, Logan, UT, USA), 1× penicillin/streptomycin/amphotericin B (Biological Industries, Haemek, Israel), 1× non essential amino acid stock solution (Sigma), 0.1% 2mM L Glutamine (Gibco

A)   B)

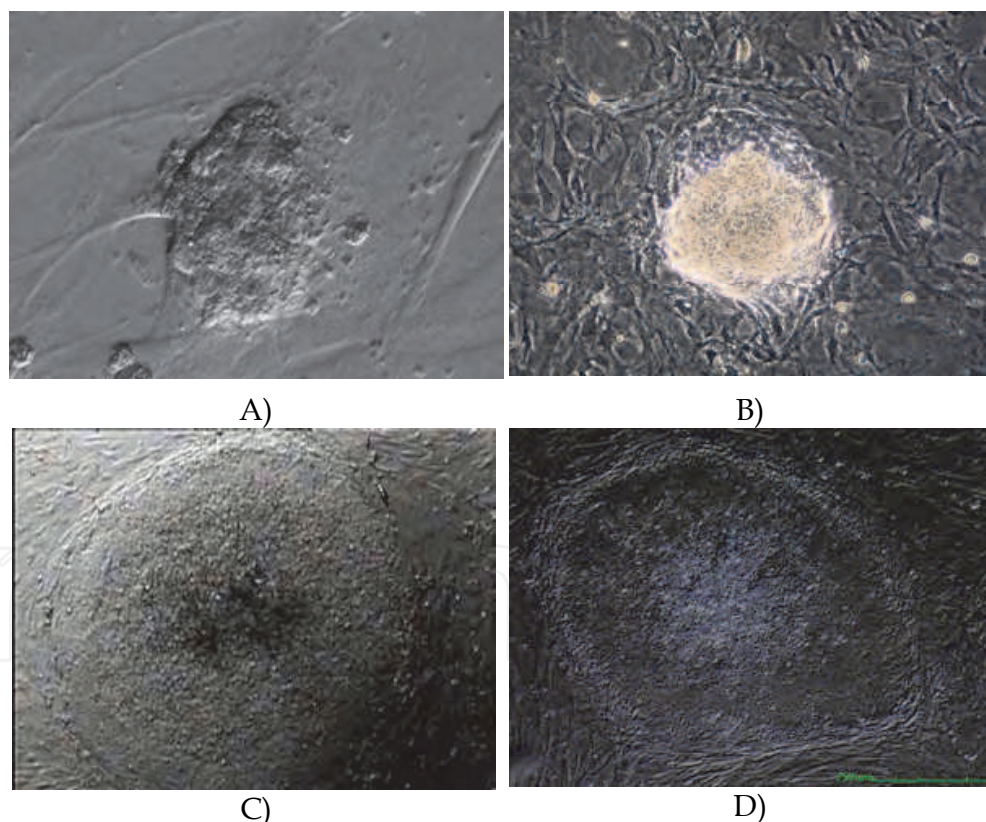C)   D)

Fig. 1. Establishment of hESC line on HFF and MEF feeder cells. Phase contrast microscopy of NS-10 line at different stages of development. **A)** The formation of outgrowth from the inner cell mass of blastocyst after direct culture of zona-free blastocyst on HFF. **B)** Cell clump, which was formed after mechanically dissociation of outgrowth, included primary hESC like cells. **C)** Circular primary NS-10 colony on MEF. **D)** Polarized colony morphology of NS-10 on HFF. Original magnifications: (A-D) X 200

BRL), 4ng/ml basic fibroblast growth factor (bFGF) (Chemicon, Temecula, CA, USA), β-mercaptoethanol (0.1 mmol/l; Sigma), and 0.1% insulin transferrin selenium complex (Gibco BRL). Culture media was changed daily.

Undifferentiated hESC colonies were split into small colony pieces mechanically with the flame-drawn glass every after 7-8 days of culture. Enzymatic dissociation of hESC was not preferred in this study. Each hESC line was cultured at least up to fifteen passages and was cryopreserved by vitrification technique according to the previously reported protocols (Reubinof et al., 2001; Vanderzwalmen et al., 2003). Briefly, colonies were first mechanically split into small pieces and were sequentially vitrified in two solutions including different concentration of DMSO and ethylene glycol.

Cryopreserved hESC lines were warmed sequentially in solutions including 0.5M and 0.25M sucrose to control efficiency of vitrification and following warming techniques (Reubinoff et al., 2001).

2.4 Karyotyping and immunocytochemistry of hESC lines

G-banding technique was used to karyotype hESC lines. HESC colonies were first incubated with culture medium including 0.1 μg / ml Colcemid (Biological Industries) for 2 h at 37 °C in a %5 CO_2. Then colonies were split into small pieces mechanically and incubated in 0.075M KCI hypotonic solution for 17 minutes at 37°C. Colonies were fixed with methanol-acetic acid solution (3:1) and processed for G-banding analysis. For each line at least 20 metaphases were analyzed for confirmation.

Karyotyping of each line was performed several times to assess whether karyotypes were stable during their long term culture. Confirmation of genetic mutation in hESC line derived from affected embryo was performed by the same procedures applied for single blastomere mutational analysis.

Surface expression markers (SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81), which are unique for undifferentiated hESC lines, were immunochytoemically analyzed according to the manufacturer's instructions (Chemicon) by using flourescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (anti-IgG) (Santa Cruz Biotechnology, California, USA). Negative controls were performed by addition of phosphate buffer saline instead of the primary antibody. All the other reagents were the same as in the slides run for specific antibodies, except nucleus of cells were stained by 4',6-diamidino-2-phenylindole (DAPI) for visualization.

Alkaline phosphatase activity (Chemicon) of hESC lines was detected by using the Chemicon Alkaline Phophatase Detection kit (Chemicon). Expressions of OCT-4 and housekeeping gene, glyceraldehyde-3 phosphate dehydrogenase (GADPH), genes were detected by reverse transcriptase PCR (RT-PCR). Briefly, extraction of RNA from undifferentiated hESCs and synthesis of cDNA were carried out by using Rneasy Mini Kit (Qiagen Gmbh, Strasse, Germany) and Sensiscript RT Kit (Qiagen), respectively. Then, PCR and following analysis were performed according to the protocol of Amit et al (Amit et al., 2002).

2.5 Differentiation potential of hESC lines

The differentiation ability of hESC lines were analyzed only by *in vitro*. For *in vitro* differentiation, embryoid bodies (EBs) were first generated according to previously published protocol (Itskovits-Eldor et al., 2000; Carpenter et al., 2003). In a brief, small pieces

of undifferentiated hESC colonies were transferred into the non adherent bacterial petri dishes and were cultured in CSCM without bFGF for 8-10 days. The resulting EB, which theoretically comprise three embryonic germ layers, were then plated on to 0.1% gelatin coated plastic petri dishes and cultured for long-term for spontaneous differentiation.

Differentiation features of hESC lines was examined under phase contrast microscope and differentiated cells were analyzed by immunocytochemical staining with markers for endoderm (cytokeratin 18, specific marker for epithelial cells, Chemicon), mesoderm (troponin I, specific for cardiac muscle; Chemicon) and ectoderm (Nestin, specific marker for progenitor of neuron, and MAP2AB, specific marker for mature neuron; Chemicon) according to manufacturer's instruction.

Additionally, in these lines (OZ, OZ-1 and OZ-2), rhythmically beating of cardiomyocytes within spontaneously differentiating embryonic stem cells were further analyzed by transmission electron microscopy (TEM). Briefly, cell clumps with spontaneous contractions were gently removed from the culture plate and fixed in 2% glutaraldehyde in 0.1 mol/sodium cacodylate buffer (pH 7.4) for 2 hours. Secondary fixation was performed in 1% OsO4 in the same buffer for 1.5h. The grids were dehydrated in graded ethanol and embedded in Epon 812. The very thin sections about 80 nm were cut and stained with lead citrate for 8 min in order to identify the cellular structures of cardiac muscle cells.

3. Results of hESC study

Experience of hESC from Memorial Hospital comprise three phrases; derivation of first hESC lines in Turkey, which was reported previously (Findikli et al., 2005), using HFF as a feeder cell instead of MEF to derive new hESC lines and derivation of hESC lines from donated embryos from PGD cycles (Candan & Kahraman, 2010).

In the first phase, nine hESC lines, which were named NS-1, NS-2, NS-3, NS-4, NS-5, NS-6, NS-7, NS-8 and MINE, were derived from 26 donated blastocysts stage human embryos with a 34.6% success rate (Table 1). Twenty blastocysts were spare IVF/ICSI embryos and 5 hESC lines (NS-1, NS-2, NS-3, NS-4 and MINE) were derived from these embryos. The remaining 6 embryos had mismatched HLA type and, therefore were not eligible for transferring in PGD cycle. From these embryos, 4 hESC lines (NS-5, NS-6, NS-7 and NS-8)

	No.of blastocysts	No. of stem cell line, n, (%)	Names of derived hESC lines
IVF/ICSI embryos	30	8, (27)	NS-1,2,3,8, MINE, and OZ, OZ-1, OZ-2
PGD for single gene disorder	8	1, (13)	OZ-8
PGD for single gene disorder & HLA typing	6	4, (67)	*NS-4, 5, 6, and 7
PGD for chromosomal screening	42	7, (17)	OZ-3, OZ-4, OZ-5, OZ-6, OZ-7, NS-9, NS-10
Immunosurgery method	15	4, (27)	NS-1,2,3,4
Direct culture method	76	16, (21)	

Table 1. A total number of embryos used for hESC lines and overall outcomes

were derived. Four lines (NS-1, NS-2, NS-3, NS-4) out of 9 hESC lines were obtained by immunosurgey and remaining 5 lines were derived by direct culture (Table 1). Two cell lines (NS-1 and NS-2) were spontaneous differentiated during their first days of *in vitro* culture.

In the second phase of hESC research in Istanbul Memorial Hospital, as an alternative to MEF, HFF was used as a feeder cell for establishment and long-term culture of new hESC lines. Three hESC lines (OZ, OZ-1 and OZ-2) were derived from 10 blastocyst stage spare IVF/ICSI embryos by the direct culture technique with a 30% success rate (Table 1). Unlike to circular colony morphology of hESC colonies on MEF, hESC colonies on HFF had angular shaped morphology, due to the polarity of HFF cells (Figure 1 C-D).

In the final phase, following PGD, embryos diagnosed as having chromosomal abnormalities and single gene mutations were used to establish hESC lines (Table 1). Forty two blastocysts with different chromosomal aneuploidies were directly placed either on MEF or on HFF. From those embryos 7 hESC lines (OZ-3, OZ-4, OZ-5, OZ-6, OZ-7, NS-9 and NS-10) were derived (Table 1 and 2). Of these 7 hESC lines, one line (OZ-3) was derived from biopsied embryo whose diagnosis was suspicious. Although chromosomal content of biopsied blastomere from this embryo was identified as abnormal (trisomy 15) by FISH, because of the fragmentations in nuclear structure of blastomere, we could not interpret the result exactly and thereby assumed it as an abnormal (Candan & Kahraman, 2010).

Three embryos diagnosed as carrying cystic fibrosis and 5 embryos with beta-thalassemia were used to isolate hESC lines with genetic disorder. However, only one hESC (OZ-8), which had a single gene mutation causing beta-thalassemia, was isolated successfully. Only 4 hESC lines were isolated by immunosurgery and the remaining hESC lines were derived after direct culture of blastocysts on feeder cells (Table 2). Following to either direct culture or immunosurgery, the developing three dimensional outgrowths from ICM were split mechanically into small clumps and transferred onto new feeder plate. Duration for successful derivation of first primary hESC colonies was ranged 15 to 20 days, based on the quality of inner cell mass of blastocysts, and application of isolation techniques properly.

Following the first several passages of primary hESC colonies, flat colonies of cells with a distinguishable compacted colony structure, well defined colony border and cellular morphology with higher nucleus to cytoplasm ratio and prominent nucleoli were obtained (Figure 1C-D). Regardless of type of feeder cells, these unique colony features were similar in all hESC. Each of hESC lines were passaged mechanically every after about 7-8d for more than 15 passages (Table 2). While passaging hESC colonies, spontaneously differentiated cells, which were observed frequently in the central or in the periphery part of colonies, were always removed mechanically to maintain undifferentiation state of colonies.

3.1 Unique features of hESC lines

All hESC lines were characterized for cell surface expression markers, which are unique to undifferentiated human embryonic stem cells. As shown in Figure 2A, established hESC lines represented a high level of alkaline phosphates activity. Furthermore, immunocytochemical staining revealed that derived 18 hESC lines were positive for SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 (Figure 2B). Negative control slides of immunocytochemical staining showed that primary antibodies specifically bound to the certain surface antigens (Figure 2C).

	NS-3	NS-4	NS-5	NS-6	NS-7	NS-8	NS-9	NS-10	MINE
Embryo source	IVF/ICSI	IVF/ICSI	PGD	PGD	PGD	PGD	PGS	PGS	IVF/ICSI
Embryo grades	4BB	3BA	4AA	4AA	4AB	3AB	4BB	4BA	4BB
PGD/PGS results	-	-	HLA mismatched	HLA mismatched	HLA mismatched	HLA mismatched	Tetraploid	Monosomy -13	-
Feeder cell	MEF	MEF	MEF	MEF	MEF	MEF	MEF	MEF	MEF
Alkaline phosphatase	+	+	+	+	+	+	+	+	+
Immuno-staining	+	+	+	+	+	+	+	+	+
SSEA-3	+	+	+	+	+	+	+	+	+
SSEA-4	+	+	+	+	+	+	+	+	+
TRA-1-60	+	+	+	+	+	+	+	+	+
TRA-1-81	+	+	+	+	+	+	+	+	+
OCT-4	+	+	+	+	+	+	+	+	+
In vitro differentiation	+	+	+	+	+	+	+	+	+
Max. passage no. of lines	35	60	39	20	22	22	21	28	49
Karyotype	46 XY	46 XX	46 XX	46 XY	46 XX	46 XX	46 XY	46 XX	46XX

	OZ	OZ-1	OZ-2	OZ-3	OZ-4	OZ-5	OZ-6	OZ-7	OZ-8
Embryo source	IVF/ICSI	IVF/ICSI	IVF/ICSI	PGS	PGS	PGS	PGS	PGS	PGD
Embryo grades	4AA	3AA	4BB	4BB	4AB	3BB	4AC	4BB	4AB
PGD/PGS results	-	-	-	Undiagnosed embryo	Trisomy 21	Monosomy 15+ Trisomy 21	Monosomy 16+ Trisomy 16	Monosomy 16	Beta-Thalassemia
Type of feeder cell	HFF	HFF	HFF	HFF	HFF	HFF	MEF	MEF	MEF
Alkaline phosphatase	+	+	+	+	+	+	+	+	+
Immuno-staining	+	+	+	+	+	+	+	+	+
SSEA-3	+	+	+	+	+	+	+	+	+
SSEA-4	+	+	+	+	+	+	+	+	+
TRA-1-60	+	+	+	+	+	+	+	+	+
TRA-1-81	+	+	+	+	+	+	+	+	+
OCT-4	+	+	+	+	+	+	+	+	+
In vitro differentiation	+	+	+	+	+	+	+	+	+
Max. passage no. of lines	19	15	15	22	17	20	22	18	18
Karyotype	46 XY	46 XX	46 XX	46 XX	46 XX	46 XY	46 XX	46 XY	46 XX

Table 2. Unique features of 18 hESC lines. (+) represents that HESC line is shown to be positive for those expression markers.
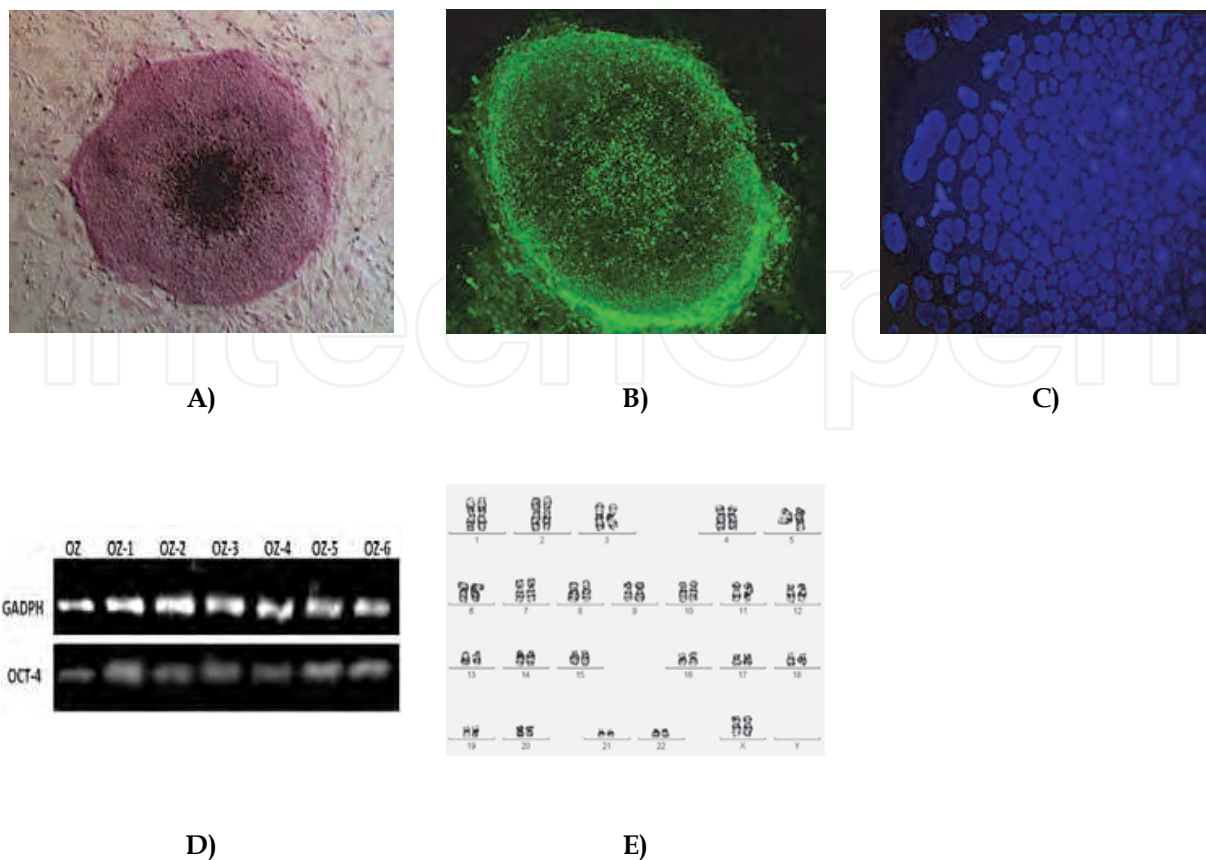
A)

B)

C)

D)

E)

Fig. 2. Unique features and karyotyping of OZ-6 hESC line. **A)** alkaline phosphatase staining, **B)** SSEA-3, SSEA-4, TRA-1-60, TRA-1-81and **C)** negative control for immunostaining of hESC lines (cells were stained by DAPI for visualization). Expression of OCT-4 and housekeeping gene GADPH in hESC lines (OZ, OZ-1-7). **E)** Karyotype analyze of OZ-6 line in passage 5, 46XX. Original magnifications: **(A-C)** X 200. Abbreviations: OCT-4, octamer-4 and GADPH, glyceraldehyde-3 phosphate dehydrogenase.

Our hESC lines were not analyzed for SSEA-1 expression, a specific marker for mouse embryonic stem cells. In consistent with the previous reports, expression intensity of SSEA-3 among hESC lines was variable and comparably weaker than SSEA-4 which was consistent and expressed higher in all hESC lines (Oh et al., 2005). Additionally, OCT-4 expressions were higher in all undifferentiated hESC lines when compared to expression level of housekeeping gene GADPH (Figure 2D).

Testing differentiation capacity of each hESC line by embryoid body formation *in vitro* revealed that these lines were capable of differentiating into various cell types derived from the three embryonic germ layers (Figure 3). Spontaneous contracting cell clumps, neural rosette structures, neural-like cells, epithelial like cells were observed under phase microscope and these differentiating cells were discriminated by immunocytochemically (Figure 3B). However, cell lines showed a relatively different capacity or tendency to differentiate into certain type of cell lineage.

Rhythmically contractions in cell clusters were started approximately after ten days from plating EBs on bacterial culture plates and kept continuing up to six weeks. During this

period, beating cell clusters, belonging to OZ, OZ-1 and OZ-2 hESC lines, were further analyzed by transmission electron microscopy. Thereby, sarcomere, intercalated disc and myofibril structures were well defined in cardiac muscle cells by TEM analysis (Figure 3E). Following vitrification and thawing procedures, all cell lines could retain their unique properties; long-term extension and pluripotency capacity *in vitro*.
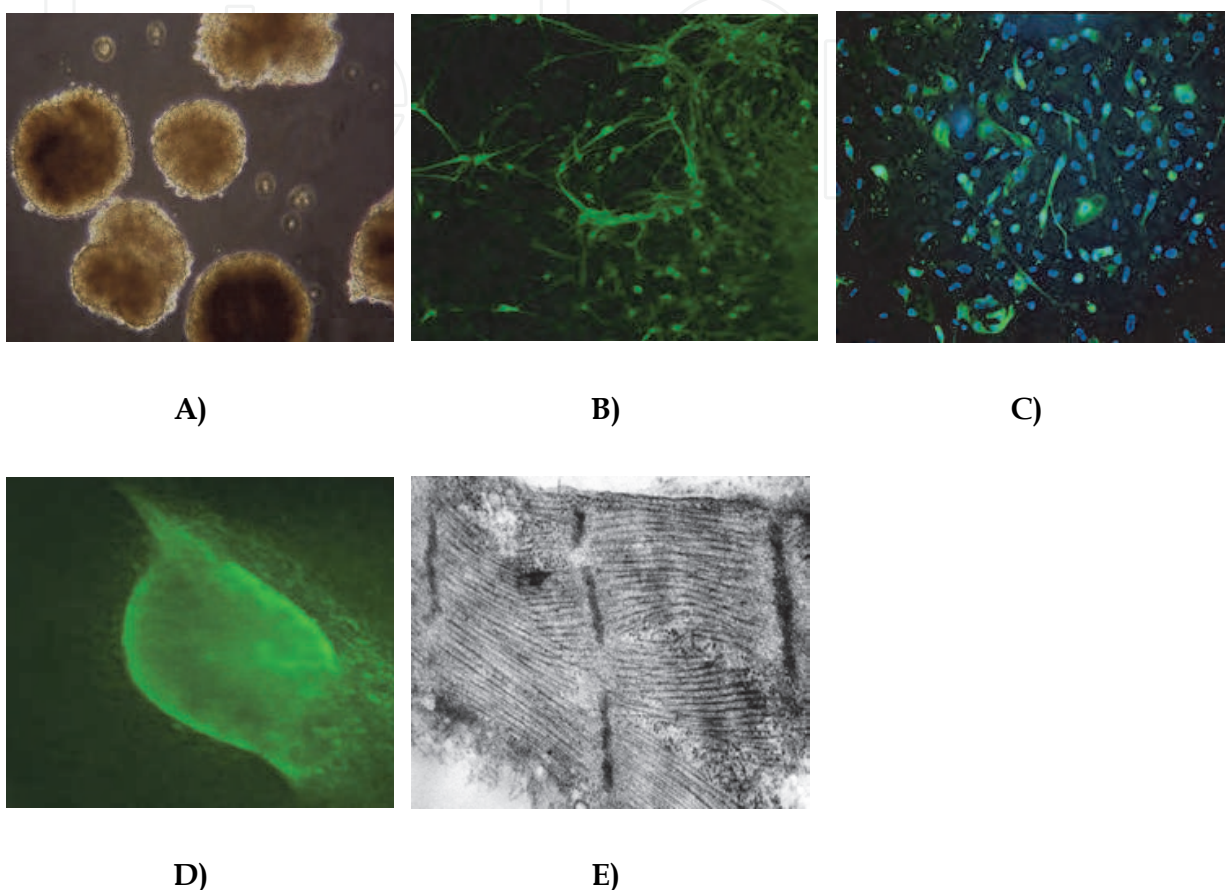

A)   **B)**   **C)**

D)   **E)**

Fig. 3. In vitro differentiation of NS-10 hESC line by embryoid body formation **A)** Phase contrast microscopy of 10-day EBs. Spontaneously differentiating cells **B)** neuron-like cells, positive for neuron specific nestin, **C)** epithelial-like cells, positive for cytokeratin 18 (green) and **D)** cardiac muscle cells, positive for troponin I. **E)** TEM photographs of beating cardiomyocytes. **(A)** X 100, **(B-D)** X200, **(E)** X12000**.**

3.2 Karyotyping and genetic analysis of hESC lines

Karyotyping analysis of all hESC lines were performed at the 5th passages. HESC lines, which were derived from supernumerary embryos after IVF/ICSI cycles and from PGD embryos, having genetic disorder and/or having mismatched HLA, had normal karyotypes (Figure 2E and Table 2).

HESC lines derived from chromosomally abnormal embryos were first analyzed by FISH at 1st passage whether they had detected chromosomal abnormality. Surprisingly, chromosomal abnormalities were not confirmed in these lines. Contrarily, analyzed chromosomes were euploid in number. Further confirmation was performed by

karyotyping at 5th passages of these lines. In consistent with results after FISH at 1st passage, karyotyping analysis revealed that in contrast to the diagnosis after PGD their karyotypes were normal (Table 2).

Karyotyping of all hESC lines were performed further passages whether cell lines retained normal karyotypes. All hESC lines had stable karyotypes.

Mutation in OZ-8 hESC cell line with beta thalassemia disorder was confirmed by PCR and subsequent sequencing procedures at passage 6. The homozygote single nucleotide transition (guanine to adenine nucleotide transition) in second exon of beta-globin gene was detected.

4. Discussion

Human embryonic stem cell is one of the most contradictory scientific issues since it was first reported by Thomson in 1998. Obviously, this ongoing dispute has been arisen from the use of human embryo for derivation of hESC. In regard to that concern, alternative methods have been proposed by several researches. However, spare embryos generated for reproductive and therapeutic treatments still remain as a main source for hESC derivation. Therefore, registering all existing lines in a database, like stem cell bank, may decrease the necessities to derive new hESC lines worldwide and eventually ethical concern may be alleviated among the public. In that regard, all these hESC lines, which had been established and characterized in Istanbul Memorial Hospital until ruling on ban on hESC research by Turkish Health Ministry, were registered to European hESCreg in 2008.

During the hESC derivation study, 86 donated embryos, which were considered insufficient for transfer and cryopreservation after IVF/ICSI cycles and were diagnosed as having genetic disorder or chromosomal aneuploidies and having mismatched HLA type after PGD cycles, were used.

The derivation efficiency of hESC lines was 20% and success rate was directly related with the quality of blastocysts. Four (NS-1, NS-2, NS-3 and NS-4) out of 20 hESC lines were successfully derived from 15 blastocysts after immunosurgery. However, two of these lines were spontaneously differentiated at the early number of passages. Remaining 16 hESC lines were isolated through direct culture of whole blastocysts on feeder cells. These two methods had comparable success rates (27% vs 21%, *p>0.05*).

All lines described in this chapter had similar colony and cellular morphology. These lines expressed unique cell surface expression markers, including SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81. They also had high level of alkaline phosphatase activity and expressed OCT-4 gene, which keeps pluripotency of hESC lines during long-term culture (Figure 2). Moreover, these 18 hESC lines were proven to have pluripotent capacity *in vitro* by EBs formation (Figure 3). Therefore, these lines have similar unique properties as previously reported existing hESC lines.

Although all lines were capable of differentiating into derivatives of three embryonic germ layers, differentiating characteristic was varied among lines. The prevalence of differentiating cardiomyocytes was higher in EBs generated from OZ-3 line, whereas higher percentage of neuron-like cells were observed in EBs generated from OZ line. The presence of specific cell lines at a various degree in differentiating cell cultures of hESC lines may be attributed to the developmental stage of embryo used for derivation, genomic and epigenetic differences.

In PGD for chromosomal screening cycles, embryos diagnosed as having chromosomal abnormalities were likely to be discarded and not to be considered eligible for transfer. In consistent with the previous results, in which it was aimed to derive chromosomally abnormal hESC lines for investigation of various aspects of early embryonic development, all our hESC lines derived from those embryos had normal karyotypes (Munne et al., 2005; Lavon et al., 2008). As stated in these studies, this unexpected result has been suggested to self correction of embryos during long-term *in vitro* culture (Hazan et al., 2008; Lavon et al., 2008).

As a result of proposed mosaicism and trisomy rescue mechanisms in developing embryos, chromosomal self correction may be occurred. In the mosaic embryos, it was suggested that euploid blastomeres can grow preferentially to abnormal cells during early embryogenesis or alternatively, these normal cells were preferentially allocated to the inner cell mass. Therefore, embryo may be subject to self correction mechanism and may evolve to a fetus with euploid chromosomal number. In trisomic rescue mechanism, it has been speculated that embryos can be corrected in terms of chromosomal number through anaphase-lag, nondisjunction, or chromosomal demolition. However, these mechanisms are still assumptions and exacts mechanisms should be proven by further studies (Hazan et al., 2008).

Furthermore, we speculated that the long-term culture may not only induce the change in chromosomal content but also may result in various genetic or epigenetic modifications in hESC lines which could eventually impair their pluripotent and self renewal capacity. However, due to inured restriction on hESC research, these derived lines could not be analyzed regarding to these aspects (Candan et al 2010).

Embryos, having single gene mutations, are of great importance, while considering as a potential source in genetic based researches on understanding the mechanisms of disease and developing new drugs (Pickering et al., 2003; Verlinsky et al., 2005). We derived one hESC line having mutation, that cause beta thalassemia disorder, from 5 donated embryos after PGD. This cell line can provide as a cell source to study the pathology of beta thalassemia and its effects on different cell types.

5. Conclusions

HESC researches have been increasing continuously since it was first isolated and characterized. Although several problems including derivation clinical grade hESC lines, risk for teratoma formation, HLA incompatibility of cells and establishment of well defined differentiation protocols of hESC lines to certain cell types have been not been solved efficiently, there is still hope for coming day in which hESC lines will be used effectively in cell replacement therapies to treat such devastating human diseases; myocardial infarcts, spinal cord injuries and diabetes. Therefore, hESC lines reported in this chapter could be potential cell source for *in-vitro* studies involving basic molecular and stem cell biology. . Moreover, as in our study, derivation of hESC from PGD embryos with intrinsic genetic content and the disease profile could be an extremely valuable source for research on genetic diseases.

In this chapter, it is aimed to summarized establishment protocols and features of first reported hESC lines in Turkey. Turkey is among the countries in which earlier hESC studies

were established first. Today, however, Turkey is one of the countries in which hESC researches were prohibited by Government. Unfortunately, so far new legislation or regulation has not been declared. However, establishment of new guideline is on the agenda of governmental institutions nowadays. Recent lifting of ban on the federal funding for hESC research in US and given permission on first clinical trial of hESC may, in fact, affect the political opinion and hopefully may inure regulations, which resume hESC researches under the control of authority and new guidelines in Turkey.

6. Acknowledgments

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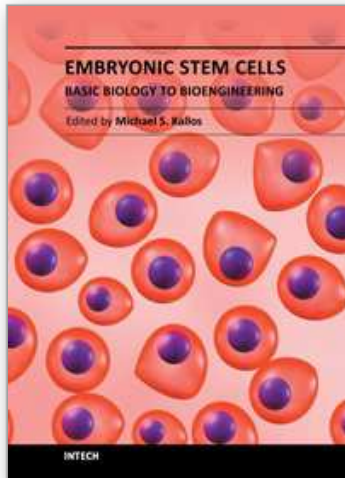
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Embryonic stem cells are one of the key building blocks of the emerging multidisciplinary field of regenerative medicine, and discoveries and new technology related to embryonic stem cells are being made at an ever increasing rate. This book provides a snapshot of some of the research occurring across a wide range of areas related to embryonic stem cells, including new methods, tools and technologies; new understandings about the molecular biology and pluripotency of these cells; as well as new uses for and sources of embryonic stem cells. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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