Derivation and Characterization of Two Sibling Human Embryonic Stem Cell Lines From Discarded Grade III Embryos

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Human embryonic stem (hES) cells are a valuable tool for studying human development in addition to their potential applications in regenerative medicine and drug discovery. The role of genetic background and epigenetic influences in development as well as in response to external influences such as drugs and therapies is well recognized. The great ethnic diversity in the Indian subcontinent translates to interindividual variability in drug response and disease susceptibility. For these reasons, new hES cell lines representing Indian genetic diversity will be valuable in studies of tissue-differentiation, cellular-function and for aspects of characterization of responses to drugs. We have derived two new hES cell lines, BJNhem19 and BJNhem20 from the inner cell mass (ICM) of discarded grade III human embryos that were not suitable for in vitro fertility treatment. Human leukocyte antigen (HLA) isotype analysis shows that they are genetically distinct from existing hES cell lines. Short tandem repeat (STR) analysis shows that the two cell lines are derived from sibling embryos. These cell lines show an undifferentiated phenotype in culture for more than 65 passages, show normal karyotype and express pluripotency markers such as TRA-1-60, TRA-1-81, stage-specific embryonic antigen-4 (SSEA-4), alkaline phosphatase, DNMT3B, GABRB3, GDF3, OCT4, NANOG, SOX2, TERF1, TDGF, LEFTA, THY1, and REX1. While both cell lines can differentiate into derivatives of all three germ layers in vitro, only BJNhem20 can form teratomas when transplanted into mice. We observe an increased frequency of cardiomyocyte differentiation from BJNhem20 embryoid bodies in feeder-free cultures upon induction with DMSO. Cardiomyocytes purified from such cultures survive and show rhythmic contractions for several weeks in culture. These hES cell lines have been accepted for deposit in the U.K. Stem Cell Bank and will be a useful resource for the international stem cell community.

Introduction

Human embryonic stem (hES) cell technology promises a new era in regenerative medicine, drug discovery and therapy. This technology, it is argued, has the potential to generate banks of specific cell types for transplantable therapy, allowing one to overcome the significant shortage of transplantable material for a wide range of human disorders [1,2]. However, despite several lines being generated in recent years, those freely available for research are still few. The routine cultivation of hES cells remains technically challenging and very demanding, justifying the need for developing novel reagents and techniques for derivation and cultivation [3]. Possible applications require standardizing of all aspects of hES cell technology including making available more information regarding the derivation process, initial propagation, differentiation, and efficient scale-up of cell lines [4].

Although several hES cell lines have been derived from across the world, only one hES cell line has been reported from the Indian subcontinent [5]. Further, the great majority of published and available lines are from Europe and North America [6] (http://www.hescreg.eu/) (http://www.mrc.ac.uk/PolicyGuidance/EthicsAndGuidance/StemCells/UsingTheUKStemCellBank/index.htm). There are

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well-documented marked differences in disease-frequency and disease-susceptibility between ethnic groups such as South Asians, Caucasians, Hispanics, African-Americans, and so on [7–10]. In this context, it may be useful to have a representation of ES cell lines representing the large and diverse Indian population. As good quality human embryos are a precious and limited resource, we have used discarded human embryos generated during in vitro fertility treatment and derived two new hES cell lines which we named BJNhem19 and BJNhem20. Human leukocyte antigen (HLA) isotype analysis and short tandem repeat (STR) analysis shows that the two cell lines are from sibling embryos. Both hES cell lines show a normal karyotype and pluripotent characteristics.

hES cells can be coaxed to differentiate in a directed fashion along specific pathways forming a wide variety of cell types derived from ectoderm, mesoderm as well as endoderm in vitro, and when transplanted into mice [11–14]. Differentiation of hES cells into specific lineages is, however, quite inefficient and much needs to be learnt, both empirically and mechanistically, about how to control and manipulate this differentiation [15,16]. Our specific interests are in differentiating hES cells to cardiomyocytes and to understand and manipulate this process. Current methods involve spontaneous differentiation of cardiomyocytes within embryoid bodies [17,18] often augmented by specific chemicals [19,20], growth factors [21,22], coculture with an endoderm-like feeder layer [23,24] or by using transgenic hES cell lines [25]. However, the success of these methods seems to depend on the specific hES cell line being used [22,26,27]. We undertook a preliminary study to assess the cardiac differentiation potential of our derived hES cell lines. We report induction of beating clusters of cardiomyocytes from BJNhem20 cells without feeder layers or external growth factors, based on modifications of reported protocols [20,28]. Further, we show that the differentiated cardiomyocytes continue to show rhythmic contractions and survive for over 2 months in culture. They continue to express cardiac markers after being isolated, expanded in culture, frozen and thawed. Hence, these cells can serve as an important hES cell-derived resource, which can be scaled up to desired numbers and frozen for future use.

Two additional hES cell lines derived from India are described in an accompanying paper from the group of Deepa Bhartiya. Together, the four hES cell lines will be a valuable addition to the existing resource available to researchers.

Materials and Methods

Derivation of hES cell lines

A total of 21 fresh discarded grade III human embryos at day 5–6, that were produced for in vitro fertility treatment but were not suitable for transfer or freezing were obtained with informed consent and institutional review board approval. The zona pellucida was removed by treatment with acidic Tyrode’s solution. Embryos were cultured whole on feeders without further manipulation or were subjected to immunosurgery using rabbit antihuman antiserum and guinea pig serum complement (Sigma Chemical Co., USA) and then plated on feeders. For some embryos, after immunosurgery, the inner cell mass (ICM) was separated from trophectoderm cells by mechanical dissection with hand-pulled micro-needles within a dish of feeders. The separated ICM was left on feeders and trophectoderm fragments were removed. For use as a feeder layer mitotically inactivated mouse embryonic fibroblasts (ATCC SCRC-1040) were plated on gelatin-coated tissue culture plates at a density of ~50,000 cells/cm². The culture medium consisted of Knockout DMEM (Invitrogen, Carlsbad, CA, USA) containing 5% Knockout Serum Replacement (Invitrogen), 5% fetal bovine serum (FBS) (HyClone; South Logan, UT, USA), 2 mM l-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids (all Invitrogen), 8 ng/mL basic fibroblast growth factor (bFGF) (Sigma), 20 ng/mL human leukemia inhibitory factor (LIF) (Sigma) and 1× antibiotic–antimycotic (Invitrogen). Between 10–14 days after plating, ICM-like clumps were mechanically dissociated and replated on fresh feeders. The resulting hES cell outgrowths were manually picked by microdissection and propagated. After the third passage, FBS was omitted from the growth medium and after 25 passages, cultures were grown without antibiotics. Cells were routinely passaged by mechanical cutting of colonies and transfer to fresh feeders. For bulk culture of cells, dishes were passaged enzymatically using trypsin or collagenase IV [3].

Cryopreservation and thawing

The cultures were cryopreserved by vitrification [29] as well as slow freezing in 10% DMSO in FBS.

Characterization of undifferentiated surface markers

Cell cultures were analyzed enzymatically or immunohistochemically for markers of undifferentiated hES cells. Alkaline phosphatase activity was assessed by incubating the cells with a 1:1 mixture of NBT and BCIP (Roche Diagnostics) according to the manufacturer’s instructions. Localization of various cell surface markers was done as described before [30] using specific antibodies to OCT4 (BD Pharmingen), stage-specific embryonic antigen-4 (SSEA-4), TRA-1–60, and TRA-1–81 (Chemicon) all at 1:50 dilution. Alexa Fluor-conjugated secondary antibodies (Molecular Probes, USA) were used at a dilution of 1:400 to immunolocalize the binding of the primary antibody.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA of 2 μg from cultured hES cell lines was reverse transcribed using Superscript II ( Gibco) and random primers, according to the manufacturer’s instructions. PCR was performed using primers specific for the different markers of undifferentiated hES cells as listed in Supplementary Table 1 (available online at http://www.liebertpub.com/scd).

Karyotype analysis

Standard G banding of metaphase spreads was performed and analyzed using CytoVision software. For each sample, 20 metaphases were counted and 15 were analyzed.
**HLA typing and STR analysis**

HLA allele typing was performed for the HLA—A, B, and DR alleles using OLRup SSP trays from GenoVision, Inc. (West Chester, PA, USA) by Manipal AcuNova Ltd., Bangalore. STR analysis was performed by Vimta Labs (Hyderabad, India) using the AmpFISTR Identifi ler Primer Set and run in ABI3130 with POP4 polymer. The data was analyzed using Genemapper ID software.

**Analysis of pluripotency in vitro**

Human ES cell colonies for each cell line were mechanically dissected out and cultured in suspension without feeders in embryoid body (EB) medium (DMEM containing 10% FBS, 2 mM l-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids) to form EBs. After 3 days, EBs were transferred to gelatin-coated dishes or glass slides and cultured for an additional 5 to 50 days to monitor spontaneous differentiation. A fraction of the EBs was allowed to continue in suspension culture to form cystic embryoid bodies (CEBs). The expression of lineage markers in the EBs was determined by staining for ectoderm, mesoderm, and endoderm. Antibodies used were against Nestin (Chemicon), β-III tubulin, Vimentin, and alpha fetoprotein (AFP) (Sigma Chemical Co., USA), Flk-1/KDR (BD Pharmingen) and Brachyury (Santacruz, USA).

**Teratoma formation**

To assess in vivo pluripotency, 1–2 million cells were harvested by trypsinization, resuspended in PBS and injected subcutaneously into immunocompromised nude mice. Mice with visible teratomas were euthanized after 8–12 weeks and the tumor was surgically removed. Analysis of differentiated tissues was done by haematoxylin and eosin staining of cryosections.

**Differentiation to cardiomyocytes**

We used a modified EB differentiation protocol with DMSO based on that described by Kehat et al. [20]. EBs were set up in cardiomyocyte differentiation medium (EB medium containing 0.75% DMSO) and at day 3, were transferred to gelatin-coated dishes. After 12 days of culture, EBs were fed every day with EB medium only. Cultures were maintained in EB medium for up to 2 months and regularly scanned for beating areas. Gene expression analysis for differentiation into cardiac lineage was studied by immunostaining for α–Actinin, cardiac Tropomyosin, and Pan-cadherin (Sigma Chemical Co., USA). Immunostaining for Nebulin was used to detect skeletal muscle cells.

**Culture, freezing, and thawing of cardiomyocytes**

Contracting clusters were mechanically isolated using microneedles, washed in microdrops of PBS, dissociated in 15 μl microdrops with trypsin/EDTA for 2–4 min at 37°C and then trypsin was inactivated and diluted out by at least 1 mL of EB medium. The entire procedure was carried out in the gelatin-coated final culture vessel to avoid losing any cells during transfer. Contracting single cells and groups of cells could be detected microscopically from the next day after plating (See supplementary movies). For cryopreservation, contracting areas were mechanically cut into pieces containing 25–50 cells and transferred to holding medium before vitrification. Cells were then passed through vitrification solutions 1 and 2 [29], taken up in straws and plunged into liquid nitrogen. Vitrified clumps were directly thawed in EB medium on to gelatin-coated dishes.

**Results and Discussion**

**Derivation of new hES cell lines**

We obtained fresh, clinically unusable, poor quality blastocyst-stage human embryos, produced by in vitro fertilization for clinical purposes, after written informed consent and approval of the Institutional Committee for Stem Cell Research and Therapy (IC-SCRT). The embryos obtained were grade III low quality blastocysts with ill-defined ICM and some irregular and fragmented blastomeres (Fig. 1A), which would normally have been discarded by the clinic as they are unusable. A total of 21 blastocysts were obtained and processed for ES cell derivation based on published protocols with some modifications [31–34]. The zona pellucida was removed by treatment with acidic Tyrode’s solution (Fig. 1B). Two embryos were cultured whole on feeders without further manipulation, one was lost during manipulation, whereas eighteen were subjected to immunosurgery using rabbit antihuman antiserum and guinea pig serum complement (Fig. 1C). Twelve embryos were plated onto feeders after immunosurgery and for the remaining six, the ICM was separated from trophectoderm cells by mechanical dissection with microneedles (Fig. 1D) and then plated on feeders (Fig. 1E). ICM outgrowth was seen from 15 embryos. However, in embryos that were plated whole or where ICM was not dissected out, the trophectoderm cells took over the culture rapidly and the ICM could not be propagated due to differentiation. Though human LIF is unable to maintain cells in a pluripotent state, hES cells do express the LIF receptor [35,36]. Comparison of published hES derivation protocols where the cell lines have maintained pluripotency over long-term culture (over 100 passages) [37], indicated that most included LIF in the medium, though the role of LIF is not yet clear. As we were modifying existing protocols for derivation from discarded embryos, we chose to include hLIF in the medium. However, once established, the cells could be successfully cultured without hLIF too. As we have to maintain uniform culture conditions over long-term passage for other experiments, we continued to include hLIF in our culture medium.

Two of the microdissected ICMs that were derived from the same batch of coded embryos on the same day, could be established as human ES cell lines (Fig. 1F–M). The hES cells have a high nucleus–cytoplasm ratio, prominent nucleoli, compact colony structure (Fig. II and M) and rapid growth rate as reported for other hES cell lines [32]. The cells are routinely passaged by mechanical dissociation of colonies and transfer to fresh feeders. For bulk culture, cells are passaged by trypsinization. Cells were frozen by vitrification at early passages and by slow freezing at later passages. Vitrification resulted in over 90% recovery after thawing.
whereas slow freezing resulted in up to 50% recovery. Derived hES cell lines have been in culture for over a year and show an undifferentiated phenotype in culture for more than 65 passages. We have named the cell lines BJNhem19 and BJNhem20.

**Characterization of BJNhem19 and BJNhem20**

The derived hES cell lines express a number of molecular markers of undifferentiated pluripotent human stem cells (Fig. 2) including octamer binding protein 3/4 (OCT3/4), SSEA-4, TRA-1-60, TRA-1-81, and alkaline phosphatase. In addition, RT-PCR analysis shows expression of DNMT3B, GABRB3, GDF3, OCT4, NANO2, SOX2, TERFI, TDGF, LEFTA, THY1, and REX1 (Fig. 3A and B). Both cell lines exhibit a normal karyotype: BJNhem19 (46, XY) characteristic of a chromosomally normal male cell line and BJNhem20 (46, XX) characteristic of a chromosomally normal female cell line (Fig. 3C and D).

HLA typing showed no common HLA-A alleles between BJNhem19 and BJNhem20. One HLA-B and two HLA-DRB alleles were matched between BJNhem19 and BJNhem20, whereas each line showed 0–1 match for HLA-B and HLA-DR alleles with the HuES9 human ES cell line [32] or the Ntera2 human embryonal carcinoma cell line [38] (Table 1). STR analysis shows that of 16 loci tested, the two cell lines share identical haplotypes at five markers and nine of the remaining markers have one allele in common. If we consider one common allele at each locus, then all alleles except D5S818 match. Further, D5S818 is homozygous in each line. These data very strongly suggest that the two cell lines are siblings (while the embryos are coded so that those involved in the derivation of the lines have no knowledge of their provenance, both lines were derived from discarded embryos on the same day). This information will be useful in the analysis of their responses to drugs or small molecules and when comparing their differentiation.

Human ES cells in culture can spontaneously differentiate into many specialized cell types such as cardiomyocytes, endothelial cells, hematopoietic cells, neuronal cells, pancreatic cells, and so on [12,14], representing all three germ layers. Both the hES cell lines efficiently formed EBs upon culture in suspension in differentiation medium. Over 50% of the EBs became fluid-filled and formed CEBs (Fig. 4A) after about 3 weeks in culture. CEB formation is a recapitulation of yolk sac development and indicative of the capacity of these cells to efficiently form early mesodermal and
endodermal lineages as well as a mature vasculature [39]. Accordingly, a well-patterned network of blood vessels could be seen in the CEBs (Fig. 4B). Immunostaining of cryosectioned CEBs showed formation of endoderm with blood vessel precursors (Fig. 4C and D). EBs that were allowed to grow attached on cell culture dishes spontaneously differentiated into neuronal, cardiovascular and endodermal lineages, for both hES cell lines. Cultures were stained at various days of differentiation and showed cells expressing the neuronal marker Nestin (Fig. 4E and F), mesodermal markers Brachyury (Fig. 4G and H) and Vimentin (Fig. 4I and J), endothelial marker VEGFR1 (Fig. 4K and L) and endodermal marker AFP (Fig. 4M and N).

To demonstrate the pluripotency of the derived hES cells, they were injected into nude mice and allowed to form teratomas. Only BJNhem20 ES cells formed teratomas. Injection of BJNhem19 cells resulted in some local proliferation of the cells but no teratoma. This suggests that though BJNhem19 behaves as a typical pluripotent cell line in vitro, it may have a limited potential for differentiation and hence cannot support teratoma formation in vivo. Some reported hES cell lines that show all properties of pluripotency in vitro are also unable to form teratomas in vivo [27,40]. Just as the requirements for differentiating different hES cell lines into specific lineages can vary in vitro [26], similarly, the ability to differentiate in vivo may also vary. In addition, the site of injection of the hES cells also affects teratoma formation [41]. Injection of larger cell numbers, in alternate sites and growth in vivo for longer duration are being attempted. The BJNhem20 teratomas showed tissues from all three germ layers (Fig. 5A–J) including skin epidermis, columnar epithelium, neural epithelium, neural rosettes (all ectoderm), smooth muscle, blood vessels, cartilage, adipose tissue (all mesoderm), gut, and glandular epithelium (both endoderm).
FIG. 3. Reverse transcription-polymerase chain reaction (RT-PCR) and karyotype analysis of undifferentiated BJNhem19 (A and C) and BJNhem20 (B and D) cells. (A and B) PCR products were obtained using primers specific for DNMT3B, GABRB3, GDF3, OCT4, NANOG, SOX2, TERF1, TDGF, LEFTA, THY1, and REX1 as indicated. Note that GABRB3 and GDF3 lanes are interchanged in (B). Abbreviation: M, molecular weight marker. (C and D) Karyotype analysis of hES cells. Shown are representative GTG-banded metaphase spreads of BJNhem19 (46, XY) and BJNhem20 (46, XX).

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<th>Table 1. HLA ISOTYPE CLASSIFICATION OF BJNHEM CELL LINES</th>
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Cardiomyocyte differentiation from BJNhem20

Directed differentiation of BJNhem19 and BJNhem20 ES cells to cardiomyocytes was done using the EB method ([18,20] and see Materials and Methods). No spontaneous or induced cardiac differentiation could be obtained from BJNhem19. While BJNhem20 cells can occasionally spontaneously differentiate into beating cardiomyocytes, addition of 0.75% DMSO to the medium and culture for 12 days gave an increased number of beating areas from day 12 to 20 of differentiation. Our preliminary analysis indicates that this protocol is robust and consistently gave contracting areas in up to 55% of EBs that could be maintained in culture for at least 2 months. Contracting areas could be detached from the EB by microneedles and maintained in suspension for at least 30 days. Detached clumps were also dissociated by trypsin and replated on glass chamber slides or 4-well dishes coated with 0.1% gelatin. Single cells as well as small clumps of 10–20 cells obtained in this way continued to beat and proliferated to large clusters of cells that showed synchronous beating (Fig. 6 and Supplementary movies). Such clusters showed morphology of cardiomyocytes and uniform staining for the cardiac markers α-actinin, cardiac tropomyosin, and pan-cadherin (Fig. 7A–C) but were negative for the skeletal muscle marker Nebulin (Fig. 7D). The majority of the dissociated cells
FIG. 4. In vitro differentiation of hES cell lines. (A and B) Bright field images of cystic embryoid bodies (CEBs) at (A) low and (B) high magnification showing the well-formed vascular network. (C and D) CEB sections immunostained to show expression of (C) AFP and (D) VEGFRII. (E–N) Fluorescent immunostaining of EBs for BJNhem19 (E, G, I, K, and M) and BJNhem20 (F, H, J, L, and N) to detect expression of ectodermal (Nestin), mesodermal (Brachyury, Vimentin, VEGFRII), and endodermal (alpha fetoprotein) markers as indicated. Scale bar: (A) 1 mm; (B–L) 100 μm.

Survived in culture on gelatin and continued beating for at least 70 days after which cell death was more apparent. Cryopreserved contracting clusters could be thawed and cultured for an additional 2 months. Clumps recovered in this manner expressed cardiomyocyte markers and continued to show rhythmic contractions.

Conclusions

In this study, we report the derivation of two new human ES cell lines from poor quality embryos that are otherwise unusable byproduct of in vitro fertility treatment. Human embryos are a precious and limited resource. The
cardiomyocytes of different genetic backgrounds. Inherent differences between hES cell lines are reported to affect the efficiency of cardiogenesis [21]. Variations in derivation and culture conditions also play a role. We show that of two simultaneously derived sibling hES cell lines, only BJNhem20 can efficiently form cardiac cells, suggesting an inherent difference [26] that can be further analyzed. Hence, the two related hES cell lines are more suitable for such comparisons than randomly chosen unrelated lines. Thus, the derived human ES cells will help understand early processes in development and also serve as a novel drug discovery tool.

Acknowledgments

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FIG. 5. Histological analysis of differentiated tissues found in teratomas formed from BJNhem20 undifferentiated hES cells. (A–J) bright field micrographs of tissues fixed with paraformaldehyde, cryosectioned (10 μ) and stained with H&E. (A) Low power image showing tissue heterogeneity within the tumor. Derivatives of all three germ layers are seen. (B–J) High power images showing various tissues formed as indicated. Square bracket in (B) indicates layers of epidermis. Arrowhead in (C) indicates eccrine sweat gland. Abbreviations: ad, adipose; ce, columnar epithelium; cg, cartilage; ec, ectoderm; end, endoderm; epi, epidermis; ge, gut epithelium; gle, glandular epithelium; me, mesoderm; ne, neural epithelium; nr, neural rosette; sm, smooth muscle tissue; v, vessel; SC, stratum corneosum; SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale. Scale bar: (A, E, and H) 250 μm, (B, C, I, and J) 100 μm, (F and G) 50 μm, (D) 25 μm.
FIG. 6. Morphology of contracting cells obtained from hES cells. (A) Beating area derived from an EB surrounded by differentiating cells. (B) Beating mass that has separated from the EB and is floating in culture. Beating mass from (B) was cut and trypsinized to dissociate cardiomyocytes and replated. Cells continued to beat and some also divided in culture. Cells imaged after (C) 1 day, (D) 7 days, (E) 50 days in culture still showed beating. (F) Isolated beating cardiomyocytes. Scale bar: (A) 250 μm, (B–D) 100 μm, (E) 50 μm and (F) 25 μm. Videos corresponding to panels E–F showing contracting cardiomyocytes are available as supplementary data.

FIG. 7. Cardiomyocyte marker gene expression. Immunohistochemical analysis of dissociated and replated cells derived from hESCs was performed for cardiac markers (A) Actinin, (B) Tropomyosin, and (C) Pan-cadherin and shows cardiomyocyte staining. Scale bar (D): 100 μm. Note that marker staining at the center of the clump is not accessible for imaging by confocal microscopy due to the thickness of the tissue.
Author Disclosure Statement

The authors declare that there are no competing financial interests.

References


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