A Modified Culture Medium Increases Blastocyst Formation and the Efficiency of Human Embryonic Stem Cell Derivation from Poor-Quality Embryos

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Abstract. Human embryonic stem cells (HESCs) are defined as self-renewing cells that retain their ability to differentiate into all cell types of the body. They have enormous potential in medical applications and as a model for early human development. There is a need for derivation of new HESC lines to meet emerging requirements for their use in cell replacement therapies, disease modeling, and basic research. Here, we describe a modified culture medium containing human recombinant leukemia inhibitory factor and human basic fibroblast growth factor that significantly increases the number of human blastocysts formed and their quality, as well as the efficiency of HESC derivation from poor-quality embryos. Culturing poor-quality embryos in modified medium resulted in a two-fold increase in the blastocyst formation rate and a seven-fold increase over the derivation efficiency in conventional medium. We derived 15 HESC lines from poor-quality embryos cultured in modified culture medium and two HESC lines from quality embryos cultured in conventional culture medium. All cell lines shared typical human pluripotent stem cell features including similar morphology, normal karyotypes, expression of alkaline phosphatase, pluripotency genes, such as Oct4, and cell surface markers (SSEA-4, TRA-1-60, TRA-1-81), the ability to form teratomas in SCID mice, and the ability to differentiate into cells of three embryonic germ layers in vitro. Our data suggest that poor-quality embryos that have reached the blastocyst stage in our modified culture medium are a robust source for normal HESC line derivation.

Key words: Derivation, Human embryonic stem cells, Modified culture medium, Poor-quality embryos

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Published online in J-STAGE: July 20, 2010
Accepted: June 11, 2010
Received: December 24, 2009

H uman embryonic stem cell (HESC) lines are typically derived from the inner cell masses (ICM) of blastocyst-stage embryos [1]. HESCs are self-renewing and have the ability to differentiate into all cell types. These characteristics provide HESCs with great potential for use in modern regenerative medicine and cell-based drug discovery.

Currently, the vast majority of HESC lines are derived from surplus cryopreserved embryos for in vitro fertilization (IVF) treatments [1, 2]. These embryos are developed to the blastocyst stage using in vitro culture prior to being plated for derivation purposes. However, the use of embryos and derivation of HESCs for research and eventual medical application has resulted in polarized ethical debates [3], since the process involves the destruction of viable developing human embryos [1]. Therefore, it is difficult to obtain good quality embryos for research purposes. During the IVF process, only a small number of all IVF zygotes will develop successfully to the blastocyst stage [3, 4]. Most of these embryos display abnormal or delayed cell division, frequently accompanied by cellular fragmentation and developmental arrest before the blastocyst stage [4, 5]. These embryos, which display poor morphology, are routinely discarded because they are unlikely to establish pregnancy. Therefore, poor-quality embryos are a more accessible and abundant source of embryos that could be used for HESC derivation. Additionally, poor-quality embryos are considered to be less ethically controversial.

Although such poor-quality embryos have been used to derive HESC lines [6–12], the efficiency of HESC derivation is very low. A major factor limiting the efficiency of HESC derivation is the poor-quality blastocysts developed from IVF of poor-quality embryos. Previous reports have shown that the addition of recombinant hLIF to the embryo culture medium could promote blastocyst development in humans [13]. Furthermore, Mitalipova et al. demonstrated that addition of LIF and bFGF to the culture medium as a mitogen could enhance the proliferation of ICM cells from blastocyst stage embryos with limited cell numbers [6]. Therefore, we hypothesized that addition of LIF and bFGF into the culture medium might improve the development of poor-quality embryos. Here, we report a modified culture medium that significantly increases the number of human blastocysts formed and their quality and the efficiency of HESC derivation from poor-quality embryos.

Materials and Methods

Source of poor quality embryos

All poor-quality embryos donated for this research were obtained from stimulated patients undergoing IVF treatment at the Reproductive Medical Center, Third Affiliated Hospital of Guangzhou Medical College. From March 2007 to May 2009, 121

(Y Fan, Y Luo and X Chen contributed equally to this work.)
couples agreed to donate their poor-quality embryos for stem cell research after being clearly informed regarding all research details at our IVF center. We obtained 419 embryos from the 121 couples. The oocyte donor average age was 32.10 ± 3.78 years. The average number of retrieved oocytes was 12.35 ± 6.78. The average number of poor-quality embryos we obtained was 3.46 ± 1.41. The study was approved by the hospital’s ethics committee.

**Embryo culture**

Embryos were morphologically graded, as described by Edwards [14] as follows: grade 1, equal-sized symmetrical blastomeres; grade 2, uneven blastomeres with < 10% fragmentation; grade 3 (10–50%), blastomeric fragmentation; and grade 4 (>50%), blastomeric fragmentation embryos. Only grade 3 and grade 4 embryos that were not used for transfer or freezing on day 3 were used after obtaining the cumulative embryo score (CES). During March 2007–March 2008, 201 day-3 poor-quality embryos were received and cultured in conventional culture medium comprised of G2.5 medium supplemented with 10% human serum albumin (Vitrolife, Sweden). During April 2008–May 2009, 218 day-3 poor-quality embryos were received and cultured in modified culture medium comprised of G2.5 medium supplemented with 2000 U/ml of human recombinant leukemia inhibitory factor (LIF, Chemicon, Temecula, CA, USA), 10 ng/ml of human basic fibroblast growth factor (bFGF, Invitrogen, Carlsbad, CA, USA) and 10% human serum albumin. The embryos were cultured for an additional 4 days at 37 C in 5% CO₂.

**Blastocyst formation**

Embryos were cultured to the blastocyst stage in two kinds of culture medium. Blastocyst quality was defined according to the serum albumin. The embryos were cultured for an additional 4 days at 37 C in 5% CO₂.

**Expression of molecular markers in HESC lines**

Total RNA was extracted from cells using the Trizol Kit (Invitrogen), and was transcribed into cDNA using oligo(dT) and Rever Tra Ace reverse transcriptase (Toyobo). PCR reactions were carried out by mixing 1 μl of cDNA template, 250 nM of each primer, 200 μM dNTP mixture and 1U of Taq DNA polymerase in a volume of 20 μl. Samples were amplified in a thermocycler. Pluripotency molecular marker genes, including OCT4 and NANOG, and marker genes for differentiated HESC, such as AFP (endoderm), NEUROD1 (ectoderm) and HBZ (mesoderm), were analyzed (Table 1). The PCR amplified products were analyzed in a 1.5% agarose gel and visualized by ethidium bromide (Invitrogen) staining.
presented in Fig. 2.

The sections were examined for the presence of tissues derived from embryos cultured in conventional culture medium (Fig. 2). The EBs were formed from each HESC line. Differentiated HESC colonies were dissociated with 1 mg/ml collagenase IV, and the colonies were transferred to 0.1% a gelatin-coated culture dish for spontaneous differentiation. The EB culture medium was equivalent to the HESC culture medium, except that it lacked bFGF and hLIF. In the modified culture medium group, 62 blastocysts were scored as grade A (Fig. 1B). In the conventional culture group, 50.2% (101/201) of the embryos were at the 2–4 cell stage, 23.4% (47/201) were at the 6–8 cell stage and 26.4% (53/201) were severely fragmented. Twenty-nine (14.4%, 29/201) embryos developed to blastocysts, and 6 (2.99%, 6/201) blastocysts were scored as grade A. All of the blastocysts were used to attempt HESC line derivations (Fig. 2).

Derivation of HESC lines

In the conventional culture medium group, 29 blastocysts were used to isolate ICMs. Of the fourteen ICMs attached to the feeder layer, 5 started to grow out. Two permanent HESC lines were obtained (FY-hES-9 and FY-hES-10; we have previously derived eight HESC lines). In the modified culture medium group, 62 blastocysts were used to isolate ICMs. Of the 39 ICMs attached to the feeder layer, 26 started to grow out. Fifteen HESC lines were produced (FY-hES-11 to 25). The derivation efficiency using day-3 poor-quality embryos cultured in conventional culture medium was significantly higher (6.9%, 15/218; P=0.00228) than for the embryos cultured in modified culture medium (10.55%, 25/218) blastocysts were scored as grade A (Fig. 1A) from March 2007–May 2009. Of these embryos, 47.3% (198/419) were at the 2–4 cell stage, 24.1% (101/419) were at the 6–8 cell stage and 28.6% (120/419) were severely fragmented. In the modified culture medium group, 44.5% (97/218) of the embryos were at the 2–4 cell stage, 24.8% (54/218) were at the 6–8 cell stage and 30.7% (67/218) were severely fragmented. Sixty-two (28.4%, 62/218) embryos developed to blastocysts, and 25 (10.55%, 25/218) blastocysts were scored as grade A (Fig. 1B). In the conventional culture group, 50.2% (101/201) of the embryos were at the 2–4 cell stage, 23.4% (47/201) were at the 6–8 cell stage and 26.4% (53/201) were severely fragmented. Twenty-nine (14.4%, 29/201) embryos developed to blastocysts, and 6 (2.99%, 6/201) blastocysts were scored as grade A. All of the blastocysts were used to attempt HESC line derivations (Fig. 2).

Characterization of HESC lines

All seventeen HESC lines exhibited AKP (Fig.1D) activity, SSEA-4, TRA-1-60, TRA-1-81, but not SSEA-1 (Fig. 3). Karyotyping of the seventeen HESC lines revealed that these lines had normal 46,XX or 46,XY karyotypes (Fig. 4). OCT4 and Nanog, which are specific marker genes for undifferentiated HESCs, were expressed in the cell lines (Fig. 5A, Table 2).

Statistical analysis

The results were compared using the SPSS 11.0 software (SPSS, Chicago, IL, USA). $\chi^2$ test (P<0.05) was used to analyze the results presented in Fig. 2.

**Results**

The effect of media conditions on blastocyst formation and quality in vitro

For the present study, our laboratory received a total 419 poor-quality embryos (299 grade 3 embryos and 120 grade 4 embryos; Fig. 1A) from March 2007–May 2009. Of these embryos, 47.3% (198/419) were at the 2–4 cell stage, 24.1% (101/419) were at the 6–8 cell stage and 28.6% (120/419) were severely fragmented. In the modified culture medium group, 44.5% (97/218) of the embryos were at the 2–4 cell stage, 24.8% (54/218) were at the 6–8 cell stage and 30.7% (67/218) were severely fragmented. Sixty-two (28.4%, 62/218) embryos developed to blastocysts, and 25 (10.55%, 25/218) blastocysts were scored as grade A (Fig. 1B). In the conventional culture group, 50.2% (101/201) of the embryos were at the 2–4 cell stage, 23.4% (47/201) were at the 6–8 cell stage and 26.4% (53/201) were severely fragmented. Twenty-nine (14.4%, 29/201) embryos developed to blastocysts, and 6 (2.99%, 6/201) blastocysts were scored as grade A. All of the blastocysts were used to attempt HESC line derivations (Fig. 2).

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In vitro and in vivo differentiation potential of HESC lines

EBs were formed from each HESC line. Differentiated HESC marker genes, such as AFP (endoderm), NEUROD1 (ectoderm) and HBZ (mesoderm), were expressed in the HESC cell lines (Fig. 5B). The results demonstrate that these cells can differentiate into three germ layers in vitro.

Teratomas were first observed 4–5 weeks after the HESCs were injected into SCID mice. Histological examination revealed that the teratomas contained the tissues of three embryonic germ layers, including the endoderm (glandular tissue), mesoderm (fat cells) and ectoderm (squamous cells; Fig. 6).

Discussion

HESCs possess a huge potential for modern regenerative medicine. To use HESCs in cell-based therapies, it is necessary to obtain enough cell lines to cover the vast spectrum of transplant...
Fig. 1. Derivation of an hESC line from grade A blastocysts after culture in modified medium (A) Representative day-3 poor-quality embryos, including the two-cell stage, four-cell stage, 6–8 cell stage and fragmentation. (B) Representative day-6 grade A blastocyst after culture in modified medium. The arrow indicates the ICM with a high number of tightly packed cells. (C) Morphology of a FY-hES-11 colony under an inverted microscope. The arrow indicates the MEFs. (D) AKP staining of FY-hES-11. The arrow indicates the MEFs do not stain for AKP. Scale bar=50 μm.

Fig. 6. Immunohistological analysis of teratomas resulting from in vivo differentiation assay using hESC lines derived from poor-quality embryos. (A) Squamous cells (ectoderm). (B) Glandular tissue (endoderm). (C) Fat cells (mesoderm). Scale bar=50 μm.

Fig. 3. Immunostaining analysis of FY-hES-11 with anti-oct4 (green), anti-SSEA-1 (green), anti-SSEA-4 (green), anti-TRA-1-60 (green), anti-TRA-1-81 (green). (A1) Oct4, (B1) SSEA-1, (C1) SSEA-4, (D1) TRA-1-60, (E1) TRA-1-81. Nuclei are stained blue with Hoechst33342 (A)-(E). Scale bar=50 μm.
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It was initially proposed that a minimum of 40–50 homozygous HESC lines would be necessary to cover approximately 50% of the human leukocyte antigen (HLA) isotypes in the American population and thus minimize the immune rejection of HESC-derived transplants [17]. More recently, the number of HESC lines needed in a stem bank for HLA matching of the UK population has been calculated to be approximately 150 [16], but this number may underestimate the lines necessary to match a very ethnically diverse population [18, 19]. Therefore, efficient derivation of more cell lines will facilitate the construction of a bank of HESC lines with diverse HLA genotypes that can serve most of the patients within a given region. Although recent reports suggest that HESCs are derived more efficiently from high-quality embryos [20, 21], it is difficult to obtain good-quality human embryos for HESC derivation. Therefore, embryos deemed

![Fig. 2](image1). The effect of LIF and bFGF on blastocyst formation from poor-quality embryos and the results of HESC line derivation. Numbers with different superscripts denote values that differ significantly within a column (P<0.05).

![Fig. 4](image2). Karyotype analysis of FY-hESCs by G-banding. (A) Normal male karyotype, 46 XY and FY-hES-11 (P=60). (B) Normal female karyotype, 46 XX and FY-hES-9 (P=40).

![Fig. 5](image3). (A) Pluripotency gene expression of FY-hES-9, 10, 11, and 17 based on RT-PCR using HUES4 expression as a control. (B) RT-PCR analysis of the expression of genes representative of the three germ layers of FY-hES-9, 10, 11 and 17 differentiated cells in vitro.
whether the blastocysts generated in the presence of LIF and bFGF of embryonic cyst attachment and implantation through facilitating upregulation to the luminal epithelium. Therefore, LIF might promote blastocyst expression in human blastocysts is important for initial attachment of a number of genes, such as ERBB4, which are involved in ICM pluripotency. Have shown that human ERBB4 protein expression in human blastocysts is important for initial attachment to the luminal epithelium. Therefore, LIF might promote blastocyst attachment and implantation through facilitating upregulation of embryonic ERBB4 [22]. It would be interesting to investigate whether the blastocysts generated in the presence of LIF and bFGF have high levels of gene expression, such as for Oct4, Sox2 and Nanog, which are involved in ICM pluripotency.

Fifteen cell lines were derived from 62 blastocysts in total, 11 from 25 grade A blastocysts and 4 from 18 grade B blastocysts. Therefore, our higher derivation efficiency may be attributed to the improved blastocyst quality. Our HESC lines shared features with HESCs, as previously reported [1, 2], including a similar morphology, normal karyotypes, expression of alkaline phosphatase and pluripotency genes, such as Oct4, and cell surface markers (SSEA-4, TRA-1-61, TRA-1-80) and the ability to form teratomas in SCID mice and to differentiate into cells of three embryonic germ layers in vitro. Specifically, two cell lines cultured beyond 60 passages maintained the same HESC marker characteristics and karyotypes exhibited in the initial cell passages. Recently, Lavon et al. reported derivation of euploid HESCs from aneuploid embryos; their results suggest that in vitro selection may occur in favor of euploid cells [23]. Aneuploidy and chromosomal mosaicism are often found in poor-quality embryos [24], yet all of the cell lines derived by our group from poor-quality embryos thus far display a normal karyotype. Our results indicate that the process of HESC derivation rigorously selects for normal ploidy.

It has been reported that poor-quality embryos, without progression to the blastocyst stage, are a poor source for HESC line derivation [9, 10, 25]. In contrast, our data suggest that poor-quality embryos that have reached the blastocyst stage in our modified culture medium are a robust source of normal HESC line derivation. In our experience, hundreds of low morphological grade and low developmental potential embryos, which are destined to be discarded, can develop to the blastocyst stage and give rise to normal new HESC lines. This finding may facilitate the possibility of establishing hESC line banks worldwide.

**Acknowledgments**

This work was supported by grants from the Guangzhou City Science and Technology Administration (2006Z1-E0021and 2008A1-E4011-3), National Natural Science Foundation of China (30871378), Guangdong Province Science and Technology Administration (2008B090500258) and Guangdong Provincial Administration (2008B090500258) and Guangdong Provincial Administration (2008B090500258).
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Medical Research Fund (B2009110).

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