Derivation of human embryonic stem cell lines from single blastomeres of low-quality embryos by direct plating

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Abstract

Purpose To explore a simple method of establishing pluripotent human embryonic stem cell (hESC) lines from single blastomeres of low-quality (LQ) embryos.

Methods Blastomeres were isolated from normally fertilized, day-3 pre-implantation LQ embryos by dissolving of the zona pellucida and were then plated directly onto inactivated human foreskin fibroblasts. The subsequent culture was identical to that used to derive a hESC line from the inner cell mass of a blastocyst. The established hESC lines were passaged and characterized.

Results Two hESC lines were produced by culturing the blastomeres individually in a hESC culture system (hESC-CS). Both of the hESC lines maintained a normal 46-chromosome XY karyotype, expressed stemness markers, and showed a pluripotent phenotype, including the ability to differentiate into all three germ layers in vitro and in vivo.

Conclusions The blastomeres of LQ embryos have a developmental capacity that necessitates prolonged culture. Plating of blastomeres from LQ embryos directly into the hESC-CS is a feasible method for deriving hESC lines.

Keywords Human embryonic stem cell · Single blastomere · Low-quality embryo · Human foreskin fibroblast

Introduction

Human embryonic stem cells (hESCs) are considered to be pluripotent and have the capacity to differentiate into

various cell types, which holds promise in the fields of biotechnology, basic scientific research, pharmacology, and cell-based medicine [1, 2]. Current challenges in hESCs research include ethical concerns, directed differentiation, and transplantation tolerance [3]. Derivation of a hESC line from a single blastomere can minimize the ethical concerns because a harmless, single-cell biopsy is used, similar to that of pre-implantation genetic diagnosis [4]. Immunological rejection is avoided when differentiated hESCs derived from a biopsied blastomere are transplanted back into the original embryo-producing individual. However, clinical applicability can only be established after intensive laboratory research delineates simple and safe derivation protocols for hESC lines. Previous studies [5–8] have described the derivation of hESC lines from single blastomeres of high-quality (HQ) embryos with sequential culture, which is somewhat complicated. Therefore, a more convenient derivation method is desired.

In vitro fertilization (IVF) produces a large quantity of low-quality (LQ) embryos that are generally discarded. While some of these blastomeres may have plasticity and latent developmental ability, to our knowledge, the developmental potential of such blastomeres has not been evaluated. This study aimed to simplify the process of deriving hESC lines from blastomeres. Here, we report the successful generation of two hESC lines from single blastomeres of distinct 5-cell stage LQ embryos by plating them directly into a hESC culture system (hESC-CS).

Materials and methods

Materials

LQ embryos were defined according to the morphological scoring method reported in 1992 and the recent Istanbul consensus for normally fertilized, day-3 pre-implantation embryos after IVF/intracytoplasmic sperm injection [9, 10].

Capsule Plating single blastomeres directly into a hESC culture system is an innovative, simple method for deriving hESC lines in vitro.

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Fig. 1 Morphology of the typical LQ embryos. LQ embryos were at 4-cell (a, 400 ×), 5-cell (b, 400 ×) and 6-cell (c, 400 ×) stages

Briefly, embryos with severe fragmentation (> 25 %), cell size not specific to the stage, or with evidence of multinucleation were graded as LQ embryos that could not be used for transfer or cryopreservation. Those LQ embryos at the 4-, 5- and 6-cell stages were included in the present study (Fig. 1). Thirty-two patients (age range, 23–41 years; mean, 31.6 ± 4.6 years) agreed to donate their LQ embryos for this study. The study was approved by the Ethical Committee of the First Hospital of Sun Yat-sen University, and was conducted with informed consent of the patients.

Cell culture and cell line derivation

Blastomeres were isolated from LQ embryos by dissolving the zona pellucida with 10 IU/mL pronase (Sigma; St. Louis, MO, USA). All intact blastomeres were then washed carefully with HEPES (Sage; Irvine, CA, USA).

For direct culture, single blastomeres were cultured individually in 48-well culture plates pre-seeded with inactivated human foreskin fibroblasts (HFFs) [11] in hESC medium consisting of 80 % Knockout Dulbecco's modified Eagle's medium, 20 % Knockout serum replacement, 1 mmol/L L-glutamine, 0.1 mmol/L 2-mercaptoethanol, 1 % non-essential amino acids, $1 \times$ penicillin/streptomycin and 2.5 μ g/L basic

fibroblast growth factor (bFGF) (Life; NY, USA). hESC-like clones were propagated and characterized as described elsewhere [12].

Characterization

Karyotyping was performed by G-banding with a Chromosome Karyotype Analysis System (Olympus; Tokyo, JPN).

The expression of cell surface markers of stemness was assessed by immunocytochemistry. The primary antibodies used were rat anti-human SSEA-3 monoclonal antibody (MA), rat anti-human SSEA-4 MA, mouse anti-human TRA-1-60 MA, and mouse anti-human TRA-1-81 MA (Millipore; Billerica, MA, USA). To detect primary antibodies, goat anti-rat IgM 488 and goat anti-mouse IgM R-PE-conjugated secondary antibodies (Life) were used.

Gene expression analyses of the stemness markers NANOG, POU5F1, REX-1, SOX-2, LIN 28, NPM1, and GDF3, and β -actin as a reference marker were carried out by qualitative reverse transcriptase-PCR (RT-PCR). RNA was isolated using an RNA Isolation Kit (Roche; Mannheim, GER) and reverse transcribed to cDNA using First-Strand Synthesis SuperMix (Life). PCR was performed using 1 μ L (1 μ g) of cDNA in a 25 μ L total PCR volume including 1 μ L

Table 1 Primer sequences and PCR conditions for stemness gene expression analyses

Gene	Forward primer	Reverse primer	Annealing temperature (°C)	Product size (bp)
NANOG	CAGAAGGCCTCAGCACCTAC	CTGTTCCAGGCCTGATTGTT	55	216
POU5F1	GACAACAATGAGAACCTTCAGGAGA	TTCTGGCGCCGGTTACAGAACCA	55	218
REX-1	GCGTACGCAAAATTAAGTCCAGA	CAGCATCCTAAACAGCTCGCAGAAT	58	306
SOX-2	CCCCGGCGGCAATAGCA	TCGGCGCCGGGAGATACAT	58	448
LIN 28	AGTAAGCTGCACATGGAAGG	ATTGTGGCTCAATTCTGTGC	58	420
NPM1	TGGTGCAAAGGATGAGTTGC	GTCATCATCTTCATCAGCAGC	58	343
GDF3	AGACTTATGCTACGTAAAGGAGCT	CTTTGATGGCAGACAGGTAAAGT	58	150
β -ACTIN	CGGATGTCCACGTCACACTT	GTTGCTATCCAGGCTGTGGT	55	469

Table 2 Primer sequences and PCR conditions for differentiation gene expression analyses

Gene	Forward primer	Reverse primer	Annealing temperature (°C)	Product size (bp)
AFP	GAAATGACTCCAGTAAACCCTGGTG	AGACTCGTTTTGTCTTCTCTTCCCC	60	400
ACTC1	CATCCTGACCCTGAAGTATCCCATC	CCCTCATAGATGGGGACATTGTGAG	60	315
SOX1	GTGTCCAATTGTTGGCATCTAGGTC	CAAGGAAATAAGGTGGTTGGAGCAC	60	202
β-ACTIN	CGGATGTCCACGTCACACTT	GTTGCTATCCAGGCTGTGGT	55	469

of each forward and reverse primer (10 μ M) (Sangon; Shanghai, CHN) and 12.5 μ L Premix Taq (TaKaRa; Dalian, CHN) on a Veriti 96-well thermal cycler (Life). The primer sequences and PCR conditions (30 cycles) are described in Table 1. PCR products were analyzed on a 2 % agarose gel.

To detect the differentiation potency in vitro, 1×10^6 cells/well were seeded in ultra-low attachment 6-well culture plates (Corning; NY, USA) and cultured in hESC medium without bFGF. The presence of all three germ layers in embryoid bodies was confirmed by RT-PCR. RNA isolation, PCR performance,

Fig. 2 Derivation of the first hESC line (S1) from a single blastomere of a 5-cell stage LQ embryo (50 % fragmentation, uneven symmetry, age 30 years). **a** The single blastomere developed into a small morula (3–4 cells) on day-2 (D2) after plating (100 $\times$). **b** On D4, the blastomere-derived morula implanted into HFFs (100 $\times$). **c** On D7, the blastomere-derived morula produced a trophectoderm (TE)-like outgrowth without showing any sign of cavitation (100 $\times$). **d** By D9, hESC-like cells were visible in the core of the outgrowth (50 $\times$). **e** These cells developed into a hESC-like clone and TE-like cells vanished in the following days (50 $\times$). **f** The clone was mechanically passaged on D12, and a typical hESC clone was observed at 7 days after the first passage (50 $\times$)

Fig. 3 Derivation of the second hESC line (S2) from a single blastomere of a 5-cell stage LQ embryo (40 % fragmentation, uneven symmetry, age 28 years). **a** Similar to the first derivation, the single blastomere split into two cells on day-1 (D1) after plating (100 ×). **b** On D3, it developed into a compacted morula (100 ×). **c** On D8, flattened TE-like cells were visible, but they did not survive in further culture (100 ×). **d–e** By D12–14, a hESC-like clone was produced (50 ×). **f** The clone was mechanically passaged on D14, and a typical hESC clone was observed at 6 days after the first passage (50 ×)

and PCR products were analyzed as described above. The primer sequences and PCR conditions (35 cycles) are described in Table 2. Teratomas were obtained after injection of 1×10^7 cells into the rear leg muscle of NOD-SCID mice aged 4–6 weeks.

Statistics

The SPSS 13.0 software package (SPSS; Chicago, IL, USA) was used for statistical analysis. The division rates of blastomeres, the rates of single blastomeres developing like pre-

implantation embryos in vitro, and the derivation rates of hESC lines between different groups were compared using the χ^2 -test. A significant statistical difference was accepted at $P < 0.05$.

Results

A total of 194 intact blastomeres were isolated from 52 LQ embryos and plated directly into the hESC-CS. Two blastomeres produced hESC-like clones without cavitations at a derivation rate of 1.0 % (Figs. 2 and 3).

Table 3 In vitro growth of single blastomeres isolated from LQ embryos

LQ embryo	Blastomere(n)	Division (%)	3-4cells(%)	Compaction (%)	Outgrowth (%)	hESC line (%)
4-cell	93	57(61.3)	39(41.9)	29(31.2)	1(1.1) ^a	0(0.0)
5-cell	50	26(52.0)	18(36.0)	10(20.0)	5(10.0) ^b	2(4.0)
6-cell	51	29(56.9)	23(45.1)	18(35.3)	2(3.9)	0(0.0)

The outgrowth rate was significantly different between a and b

The developmental stage of single blastomeres and their in vitro growth are described in Table 3. The rate of TE-like outgrowths derived from blastomeres of 5-cell stage LQ embryos was significantly higher than that of the 4-cell stage LQ embryos. However, the outgrowth rate of 6-cell stage

blastomeres was not significantly different from that of 4-cell and 5-cell stage blastomeres.

The two putative hESC lines (S1 and S2) maintained a colony morphology and growth pattern similar to that of a blastocyst inner cell mass (ICM)-derived hESC line in

Fig. 4 G-banding results of S1 and S2 cells. All cells of S1 (a) and S2 (b) cell lines displayed a normal male karyotype (46, XY)

Fig. 5 Pluripotency marker expression of S1 and S2 cells as detected by immunocytochemistry. S1: SSEA-3 (**a**), SSEA-4 (**b**), TRA-1-60 (**c**) and TRA-1-81 (**d**). S2: SSEA-3 (**e**), SSEA-4 (**f**), TRA-1-60 (**g**) and TRA-1-81 (**h**)

subsequent propagation. At passages 10 and 30, the cells were collected for karyotyping, showing a normal male karyotype (46, XY) by G-banding (Fig. 4). Both cell lines expressed typical stemness markers (SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, NANOG, POU5F1, REX-1, SOX-2, LIN 28, NPM1 and GDF3) as detected by immunocytochemistry or RT-PCR (Figs. 5 and 6). In vitro pluripotency was confirmed by the presence of all three germ layers in embryoid bodies that were analyzed by RT-PCR at 4 weeks of culture: SOX1 (ectoderm), ACTC1 (mesoderm) and AFP (endoderm) (Fig. 7). A pluripotent phenotype in vivo was shown by immunohistochemistry that was performed on teratomas generated at 8 weeks after injection (Fig. 8).

Furthermore, both S1 (passage 52) and S2 (passage 43) cells were successfully frozen-thawed and re-introduced into culture.

Discussion

We successfully established two hESC lines from single blastomeres of distinct 5-cell stage LQ embryos by plating them directly into a hESC-CS without sequential culture. Both hESC lines maintained a typical hESC morphology and growth pattern, displayed a normal karyotype, expressed pluripotency markers, and had the ability to differentiate into three germ layers both in vivo and in vitro.

Fig. 6 Gene expression of pluripotency markers in S1 and S2 cells as detected by RT-PCR. Both S1 (**a**) and S2 (**b**) cell lines expressed stemness markers: NANOG, POU5F1, REX-1, SOX-2, LIN 28, NPM1 and GDF3 (lanes 1–7). β -actin was used as a reference marker (lane 8). No primer and no template PCR control was negative (lane 9)

Currently, a widely used morphological grading method is applied in most IVF centers to select HQ embryos for transfer. The LQ embryos are discarded. However, this grading method cannot precisely predict the developmental potential of embryos, especially the potential of each blastomere [13, 14]. Veiga et al. [15] reported that pregnancy can be achieved by replacing a frozen-thawed embryo with less than 50 % intact blastomeres, indicating the full developmental potential of the blastomeres. In recent years, LQ embryos had been found to have a much higher rate of aneuploidy than that of HQ embryos. However, aneuploidy did not occur in all blastomeres of LQ embryos; most of these embryos were found to be mosaic. Therefore, LQ embryos contained euploid blastomeres that might have developmental potential and could possibly be used for the establishment of hESC lines. On the other hand, the development of cleavage embryos is mainly controlled by maternal mRNA, proteins, and mitochondria stored in oocytes before activation of the embryo genome; this material might distribute unevenly into blastomeres during the cleavage process, especially for LQ embryos. Such uneven distribution would affect the development of embryos and some of the blastomeres, but a few blastomeres might receive a full complement of maternal information, allowing further development in vitro.

Fig. 7 In vitro pluripotency of S1 and S2 cells as confirmed by RT-PCR. Embryoid bodies obtained from S1 (**a**) and S2 (**b**) cells showed the presence of cells from all three germ layers: ectoderm (lane 2: SOX1), mesoderm (lane 3: ACTC1) and endoderm (lane 4: AFP). β -actin was used as a reference marker (lane 5). No primer and no template PCR control was negative (lane 6)

Van et al. [16] have shown that single blastomeres isolated from human embryos can develop into blastocysts. Our study showed that blastomeres could produce TE-like outgrowths, without cavitation, by direct culture. Blastomeres change their phenotype from nonpolar to polarized during embryonic development. This polarization is followed by two rounds of division in which cells are allocated to different positions and phenotypes: differentiative divisions generate nonpolar inner and polar outer cells, while conservative divisions generate two polar outer cells. Thus, the ratio of inner to outer cells critically depends on the relative numbers of each division type, which is affected by cell shape and cell-cell interactions [17]. A single blastomere-derived hESC line might be achieved by suppression of polarization or, upon polarization, by maximizing the number of differentiative divisions so that the ratio of nonpolar epiblasts to polar trophoblast cells is maximized [18]. Accordingly, we considered that the culture conditions might influence the cell shape and interactions that affect the division type upon blastomere polarization. The ratio of ICM to TE determined by the division type is related to cavitation. When the ratio is high enough, blastomeres produce a larger ICM, which favors establishment of hESC lines without the incidence of cavitation due to the low number of TE cells. In our study, it appeared that plating blastomeres directly

Fig. 8 In vivo pluripotency of S1 and S2 cells as shown by immunohistochemistry. Teratomas obtained from S1 (a, 200 $\times$) and S2 (b, 200 $\times$) cells showed the presence of cells from all three germ layers: ectoderm (a1 and b1: squamous epithelium), mesoderm (a2 and b2: cartilage) and endoderm (a3 and b3: glandular epithelium)

into a hESC-CS could avoid cavitation, which might facilitate a relatively high derivation rate.

Chung et al. [19] described the derivation of embryonic stem cell lines from single mouse blastomeres in 2005. The following year, these authors derived hESC lines from single blastomeres of thawed 8-cell stage embryos by co-culture with an established fluorescently-labeled hESC line, with a success rate of 2 % [5]. In addition, the derivation rate was increased to 20 % by co-culturing the blastomeres with the parent embryo and the addition of laminin to the medium [6]. All of these studies used blastomeres of HQ embryos, sequential culture, and co-culture. By 2009, the protocol was simplified by omitting the co-culture. Ilic et al. [7] derived hESC lines from blastomeres on HFFs instead of mouse embryonic fibroblasts with minimal exposure to xenomaterials, with a success rate of 12.5 %. Geens et al. [8] also reported the derivation of hESC lines from single blastomeres of 4-cell stage embryos with the same success rate. Both of these studies

used HQ embryos and sequential culture. In contrast, we used LQ embryos to derive hESC lines. LQ embryos are a conventional source of embryos for research purposes and are easily accessible from commonly used assisted reproductive technology. Nonetheless, comparison of our derivation rate with those of other studies is difficult, as most of the blastomeres of the LQ embryos were aneuploid or unevenly divided, with a much lower developmental potential. Although we found that blastomeres from 5-cell stage embryos had a higher rate of hESC line derivation than did 4-cell and 6-cell stage embryos, it is hard to draw conclusions from our data, as the sample was relatively small. However, we simplified the derivation method by plating the blastomeres of LQ embryos directly onto HHFs without sequential culture and successfully established two hESC lines, which was the main aim of the present study.

One limitation of this study is that we plated single blastomeres of human LQ embryos into the hESC-CS without precisely determining the cell populations of the ICM and TE. Immunocytochemistry can distinguish these two cell populations, which would more accurately determine the relationship between the ICM to TE ratio and cavitation. In summary, we successfully generated hESC lines using a more convenient method without sequential culture. However, the derivation rate was very low for single blastomeres of LQ embryos. In a future study, we would like to further our investigation by applying this method to HQ embryos.

Conclusion

Our study has shown that some blastomeres of LQ embryos have developmental potency. Prolonged culture of LQ embryos would be more advantageous than abandonment, and a more accurate system for embryo scoring is required. This study also demonstrates that plating single blastomeres directly into a hESC-CS is an innovative, simple method for derivation of hESC lines in vitro.

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