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Analysis of Blastocyst Culture of Discarded Embryos and its Significance for Establishing Human Embryonic Stem Cell Lines

Fang Wang,[1] Hui-Juan Kong,[1] Quan-Cheng Kan,[2] Ju-Yan Liang,[1] Fang Zhao,[1] Ai-Hong Bai,[1] Peng-Fen Li,[1] and Ying-Pu Sun[1*]

[1]*Reproductive Medical Center, First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China*

[2]*First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China*

ABSTRACT

In recent years, applications of stem cells have already involved in all domains of life science and biomedicine. People try to establish human embryonic stem cell lines (hESCs) in order to carry out hESC-related studies. In this study, we explored what embryos are conducive to the establishment of hESCs. The discarded embryos from in vitro fertilization–embryo transfer (IVF–ET) cycles were sequentially incubated into blastocysts, and then the inner cell mass (ICM) was isolated and incubated in the mixed feeder layer. The cell lines which underwent serial passage were identified. After a total of 1,725 discarded embryos from 754 patients were incubated, 448 blastocysts were formed with 123 high-quality blastocysts. The blastulation rate was significantly higher in the discarded embryos with non-pronucleus (0PN) or 1PN than in the discarded embryos with 2PN or ≥3PN. The blastulation rate of the D3 embryos with 7–9 blastomeres was higher. Among the originally incubated 389 ICMs, 22 hESCs with normal karyotype were established, and identified to be ESCs. Therefore, in establishing hESCs with discarded embryos, D_3 0PN or 1PN embryos with 7–9 blastomeres should be first selected, because they can improve high-quality blastulation rate which can increase the efficiency of hESC establishment. J. Cell. Biochem. 113: 3835–3842, 2012.

KEY WORDS: HUMAN EMBRYONIC STEM CELL LINES; BLASTOCYSTS; DISCARDED EMBRYOS

In recent years, with the development of modern medicine and aging of the population, the demand for organ donation is greatly increasing. However, there is a serious shortage of donor organs, and even though the organs are available, patients have to be faced with a problem of immunosuppressive drugs. Lifelong usage of immunosuppressive drugs will lead to complications in these patients. These problems make scientists explore new methods instead of organ transplantation. Therefore, a new field, tissue engineering, occurred in 1960s. Human embryonic stem cell lines (hESCs) were first isolated from inner cell mass (ICM) in 1990s [Thomson et al., 1998]. hESCs, pluripotent cells, are obtained by in vitro culture of blastular ICM or primordial germ cells under restraining differentiation. hESCs are good transgene carriers because they possess the following properties: multi-directional differentiation potential, undiferentiated proliferative capacity during long-term in vitro culture, maintaining normal diploid karyotype and easy gene modification. hESCs are good seed cells of tissue engineering because they can differentiate into all types of cells.

hESCs have great value in basic research and a vast prospect in clinical application due to their properties. Firstly, hESCs provide a new mode to study human normal development; based on this, we can explore abnormal pathological development and its mechanism. Secondly, hESCs provide a new research system for detection of human cell gene function. Thirdly, hESCs can provide cells, tissues, and organs for clinical transplantation, because they possess the potential of directed differentiation in vitro [Kaufman et al., 2001]. Therefore, hESCs are expected to provide an unlimited cell source for the treatment of human refractory diseases [Olson et al., 2011].

In recent years, applications of stem cells have already involved in all domains of life science and biomedicine. People try to establish hESCs in order to carry out hESC-related studies.

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*Correspondence to: Ying-Pu Sun, Number one, Constructive East Road, Zhengzhou City, China.
E-mail: syp2008@vip.sina.com

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However, there has been considerable debate about the source of hESCs. The application of human high-quality embryos in researches is restricted by ethics in many countries. Since hESCs easily differentiate spontaneously and hardly maintain normal diploid karyotype during passage, the overall efficiency of hESC establishment is low. In many Chinese Reproductive Medical Center, the discarded embryos after in vitro fertilization–embryo transfer (IVF–ET) and intracytoplasmic sperm injection (ICSI) are a important source of hESCs. In this study, we collected these discarded embryos to establish hESCs. We observed the rate and quality of blastocyst formation from different embryos in order to make the best of discarded embryos to establish hESCs. The hESCs we established and identified. This study has important significance for the establishment of large-scale hESCs.

MATERIALS AND METHODS

All study methods were approved by Institutional Review Board and Ethics Committee of the First Affiliated Hospital of Zhengzhou University. All the subjects enrolled into the study gave written formal consent to participate.

FRESH DISCARDED EMBRYOS

Fresh discarded embryos were collected from patients undergoing IVF/ICSI cycle in our Reproductive Medicine Center between June 2007 and July 2010. The fresh discarded embryos include: (1) the embryos of abnormal fertilization with non-pronucleus (0PN), one pronucleus (1PN) or multi-pronuclei ($\geq$3PN, mainly 3PN); (2) the cleavage-arrested embryos with 2 pronucleus (2PN) which were not in accord with the standards of transplantation or freezing.

CULTIVATION OF FRESH DISCARDED EMBRYOS

D_2 or D_3 embryos which were not in accord with the standards of transplantation or freezing were collected, and then placed into the droplets which were balanced overnight and covered with mineral oil to incubate at 37°C in an atmosphere of 5% CO_2 until D_5–D_7. The medium was changed and the embryos were observed daily. The quality of embryos was evaluated with Perter embryo scoring system [Brinsden, 1999]. The quality of blastocysts was characterized by the following items: blastocyst score with a range of 1–6 grades (grade 1: early blastocyst, the blastocyst cavity is less than a half of bulk volume of blastocyst; grade 2: the blastocyst cavity is more than a half of bulk volume of blastocyst; grade 3: completely expanded blastocyst, the blastocyst cavity completely occupies the hole blastocyst; grade 4: post-expanded blastocyst, the blastocyst cavity is large and the zona pellucida becomes thin; grade 5: blastocyst is hatching from the rift of zona pellucida; grade 6: blastocyst is completely outside the zona pellucida), scoring for ICM (grade A: cell number is more and cell conjugation is tight; grade B: cell number is less and cell conjugation is loose; grade C: few cells are seen) and scoring for trophectoderm (grade A: cell number is more and cells distribute around the blastocyst; grade B: cell number is less and loose; grade C: few cells are seen) [Gardner et al., 2000]. In this study, the blastocysts with grade 3BB or over were regarded as high-quality blastocysts.

ESTABLISHMENT OF hESC LINES

Preparation of the mixed feeder layer. Mitomycin C-inactivated human foreskin fibroblasts [Huang, 2003] and mouse embryonic fibroblasts [Xue, 2001] were mixed at a rate of 1:1, and then the dishes were covered with mixed feeder layer in a density of $0.55 \times 10^5/cm^2$ for future use.

Isolating ICM with the mechanical method. Trophectoderm cells around the ICM were removed with a syringe needle under a dissecting microscope, avoiding damage to the ICM as carefully as possible. The ICM was seeded in the mixed feeder layer. Cell passage was performed during 3–7 days in primary clone; and then each cell passage was performed every 4–7 days. The status of adherence and growth was observed and half of culture medium was changed 48 h after passage. Finally, these clones were incubated in H-DMEM (Gibco) containing 20% serum replacement (Gibco), penicillin/streptomycin (Gibco), basic fibroblast growth factor (bFGF; Invitrogen), insulin transferrin selenium (ITS; Gibco), non-essential amino acids (Hyclone), β-mercaptoethanol (Sigma), L-glutamine (Sigma) at 37°C in an atmosphere of 5% CO_2.

Passage performed with the mechanical method. The pyknotic and solid clones were selected, and then the needle of 1 ml-syringe was used to mechanically cut these clones. The passage ratio was 1:3–1:5.

IDENTIFICATION OF hESCs

Alkaline phosphatase (AKP) staining. hESCs were fixed with 4% paraformaldehyde for 30 min, washed with PBS (Hyclone) three times, treated with TSM1 (0.1 M Tris, 0.1 M NaCl, 10 mM $MgCl_2$, 1.0 M HCl, pH: 8.0) and TSM2 (0.1 M Tris, 0.1 M NaCl, 50 mM $MgCl_2$, 1.0 M HCl, pH: 9.5) for 10 min, respectively, washed with PBS three times again, and visualized with AKP color reagent (NBT/BCIP; Vector) at room temperature in the dark for 2 h. The status of coloration was observed under the microscope. hESCs were washed with distilled water. Following glycerin mounting, hESCs were observed.

Immunofluorescence. hESCs were fixed with 4% paraformaldehyde for 30 min, washed with PBS three times, and treated with 0.1% Triton-X-100 at room temperature for 5 min. After that, the clones were placed into PBS containing 3% BSA for 30 min, and then 1:40 (v:v) of SSEA-4, SSEA-1, NANOG, SOX2, and TRA-1-60 as first antibody (Santa Cruz) were added for incubation at 4°C overnight. The next day, the corresponding secondary antibodies (Beijing Zhongshan) were added for incubation at 37°C for 30 min followed by observation under the fluorescence microscope.

Expression of transcription factor OCT-4 detected with RT-PCR. RNA was extracted with Trizol from hESCs to synthesize cDNA through reverse transcription, and then cDNA underwent RT-PCR amplification. PCR reaction system was carried out in a volume of 50 μl containing 5 μl of 10× PCR buffer, 4 μl of 2.5 mM dNTPs, 1 μl of each primer, 0.5 μl of Taq enzyme, 8 μl of cDNA template, 31.5 μl of ultra-pure water. RT-PCR reaction conditions were as follows: pre-denaturing at 94°C for 2 min, denaturing at 94°C for 45 s, reannealing at 55°C for 45 s, elongation at 72°C for 45 s, 30 cycles; finally elongation at 72°C for 5 min. PCR products were stored at 4°C. The PCR products underwent agarose gel electrophoresis, and then the electrophoretic results were analyzed with an ultraviolet-

automatic image analyser. Detailed procedures were strictly carried out according to the kit instructions. Primer size was 242 bp, with a sequence of 5′-GCGACTATGCACAACGAG-3′ and 5′-GAGTGATGACGGAGACTGG-3′.

Expression of transcription factor OCT-4 detected with Western blot. After extracted from 40-passage hESCs, the protein underwent SDS–PAGE electrophoresis, and then was transferred to a nitrocellulose membrane. The membrane was sealed with TBST containing 10% of non-fat dry milk for 30 min followed by addition of 1:500 of rabbit anti-human OCT-4 antibody for 3-h incubation at room temperature. The membrane was washed with TBST three times followed by addition of HRP-conjugated mouse anti-rabbit secondary antibody for 2-h incubation. The membrane was washed with TBST three times and washed with PBS once followed by coloration with ECL luminescence reagent for 20 s. The membrane was exposed for 5 min, visualized for 20 s, fixed for 20 s, and washed for 5 min followed by observation.

In vitro differentiation into embryoid bodies. Cells were digested with trypsin, blown into single cell suspension, and adjusted at a concentration of 4×10^4/ml. Every hanging drop containing 30 μl of suspension was placed on the dish cover, and then incubated at 37°C in an atmosphere of 5% CO_2. Three days later, simple embryoid bodies were formed, transferred into Petri dishes for incubation in bFGF-free culture medium, and blown into cell suspension. These simple embryoid bodies were taken out once or twice a day to shake gently. The medium was changed when necessary. Five to 10 days later, cyst-shaped embryoid bodies could be seen.

Differentiate into teratomas in vivo. hESC clones were mechanically cut into 50–100 small cell aggregates which were inoculated into the hind legs or groin of SCID mice (Beijing Vital River Laboratory Animal Co. Ltd., Beijing, China). Eight to 12 weeks later, when the tumor diameter reached 1 cm, the tumor was taken, washed with PBS, fixed with 10% paraformaldehyde, embedded in paraffin followed by slicing and HE staining. finally the samples were observed under microscope.

Chromosome G-banding karyotype analysis. The hESCs were placed in 1.5 ml of culture medium containing 30 μl of colchicine (with a final concentration of 0.2 μg/ml) to incubate at 37°C in an atmosphere of 5% CO_2 for 3 h, washed with PBS twice, digested with 0.05% trypsin, and blown into single cell followed by stopping digestion. The supernatant was removed by centrifugation at a rate of 3,000 rpm followed by addition of 1.5 ml of KCl hypotonic solution (0.075 mol/L) for 15 min. A few drops of stationary liquid (methanol:acetic acid = 3:1) were added for pre-fixation, following removal of the supernatant through centrifugation, 1 ml of stationary liquid was respectively added to treat for 40 and 20 min. After removal of the supernatant through centrifugation, two to three drops of stationary liquid were added and well mixed, and the sample sections were made. After atmospheric drying, the sample sections were baked at 90°C for 30 min, treated with 0.05% trypsin for 3–10 s, quickly washed twice with saline, stained with Giemsa solution for 10 min, and washed with distilled water. After atmospheric drying, these sections were observed and analyzed under an oil immersion lens.

STATISTICAL ANALYSIS

Statistical analysis was performed with SPSS11.0 software. Data are expressed as percentage. Chi-square test was used in comparison between percentages. Test standard was set at $\alpha = 0.05$, and $P < 0.05$ was considered significant.

RESULTS

BLASTULATION STATUS IN DIFFERENT EMBRYOS

In this study, a total of 1,725 discarded embryos from 754 patients who signed the informed consent were sequentially incubated into 448 blastocysts containing 123 high-quality blastocysts. The blastulation rate was the highest in the embryos with 1PN ($P < 0.05$); in the embryos with 0PN, the blastulation rate was the second. The blastulation rate was significantly higher in the embryos with 1PN or 0PN than in the embryos with 2PN or ≥3PN. There were no statistical differences in the rates of high-quality blastocysts between different embryos (Table I).

THE RELATIONSHIP BETWEEN THE NUMBER OF D_3 EMBRYONIC BLASTOMERES AND BLASTULATION

Blastulation rate in D_3 embryos with 7–9 blastomeres was higher than that in D_3 embryos with ≤6 blastomeres ($P < 0.05$), similar to that in D_3 embryos with ≥10 blastomeres without statistical difference. Blastulation rate was the lowest in D_3 embryos with ≤3 blastomere ($P < 0.05$). There were no statistical differences in the rates of high-quality blastocysts between different embryos (Table II).

COMPARISON OF hESC ESTABLISHMENT BETWEEN DIFFERENT QUALITY BLASTOCYSTS

The adherence rate, passage rate, and hESC establishment rate of high-quality blastocysts were all significantly higher than that of low-quality blastocysts ($P < 0.05$; Table III).

ESTABLISHMENT OF hESC LINES

After the successful exploration of the factors affecting the establishment of hESC lines, a total of 22 hESC lines were established, and passaged to over 20 generations. The 22 hESC lines have been identified, and were called ZZU-hESC-1, ZZU-hESC-2 . . . ZZU-hESC-22, respectively, according to the order of hESC establishment.

IDENTIFICATION OF hESCs

Morphology of growth. Under inverted microscope, hESCs grew in colony, and the shapes of these colonies were flat with clear cell boundaries (Fig. 1). In these colonies, the nuclei of these stem cells

TABLE I. Blastulation Status in Different Embryos

Group	No. of embryos (n)	Blastulation rates (%)	Rates of high-quality blastocysts (%)
0PN	424	23.82* (101/424)	20.79 (21/101)
1PN	629	41.65* (262/629)	33.58 (88/262)
≥3PN	359	13.64 (49/359)	16.32 (8/49)
2PN	313	11.50 (36/313)	16.67 (6/36)

*$P < 0.05$, when compared with other three groups.

TABLE II. The Relationship Between the Number of D_3 Embryonic Blastomeres and Blastulation

No. of blastomeres (n)	No. of embryos (n)	Blastulation rates (%)	Rates of high-quality blastocysts (%)
≤3	338	6.21* (21/338)	9.52 (2/21)
4–6	669	25.11 (168/669)	26.79 (45/168)
7–9	501	46.51** (233/501)	30.47 (71/233)
≥10	86	30.23 (26/86)	19.23 (5/26)

*$P < 0.05$, when compared with other three groups.
**$P < 0.05$, compared with above two groups.

were large with clear nucleolus and high nucleoplasmic ratio. hESCs reached the growth peak on D_4–D_6, and began differentiating on D_7.

AKP staining. hESCs with AKP expression were violet-blue (strongly positive), while the feeder layer cells failed to be stained (Fig. 2).

Immunofluorescence. hESCs were positively stained for SSEA-4, NANOG, and TRA-1-60 in green fluorescence and positively for SOX2 in red fluorescence, but negatively for SSEA-1. Feeder layer cells failed to be stained (Fig. 3).

Expression of cell-specific gene. RNA was extracted from hESCs. The expression of cell-specific gene was detected with RT-PCR, and results showed the high expression of transcription factor OCT-4 to maintain stem cell pluripotency and self-renewal (Fig. 4).

Expression of transcription factor OCT-4 protein. OCT-4 protein was detected with Western blot, and results indicated high expression of OCT-4 protein in hESCs (Fig. 5).

In vitro differentiation into embryoid bodies. Simple embryoid bodies were formed after hESC were cultured for 3 days with the hanging drop method. The simple embryoid bodies were transferred into 10-cm dish to incubate for 5–7 days, the sizes of simple embryoid bodies (Fig. 6A) were gradually increased and cyst-shaped embryoid bodies (Fig. 6B) occurred.

Differentiation into teratomas in vivo. The hESC cells were injected into the hind legs or groin of SCID mice. 8–12 weeks later, tumor-like tissue was seen without marked peplos, and tumor-surrounding tissue was squeezed without marked destruction. The tumor was taken, fixed with 10% paraformaldehyde, embedded in paraffin followed by slicing and HE staining. The tumor contained glands (Fig. 7A), muscle tissue (Fig. 7B), fat cells (Fig. 7C), bone, etc., demonstrating that hESCs may developed into three germ layers-derived teratoma in vivo (Fig. 7).

TABLE III. Comparison of hESC Establishment Between High-Quality Blastocysts and Low-Quality Blastocysts

Items	High-quality blastocysts	Low-quality blastocysts
No. of ICM (n)	103	286
Adherence rate (%)	84.47% (87/103)*	58.39% (167/286)
Passage rate (%)	43.68% (38/87)*	40.71% (68/167)
hESC establishment rate (%)	19.42% (20/103)*	0.70% (2/286)

ICM, inner cell mass.
*$P < 0.05$, when compared with low-quality.

Fig. 1. hESC clones under inverted microscope (4×, ZZU-hES-1, Passage 37) hESCs show nest-like growth with clear cell boundaries. Cell volume is small, but nuclei are large with high nucleoplasmic ratio.

Karyotype analysis. karyotype analysis was performed every five generations in the 22 hESCs. All karyotypes were normal (Fig. 8) with 46XX in 12 hESCs and 46XY in 10 hESCs.

DISCUSSION

There are differences in many aspects such as cloning efficiency, doubing time and differentiation tendency between the hESCs which have been established in the world. These differences also have occurred in the hESCs we established. These results demonstrate that besides the stem cell-related general characteristics, different hESC lines also possess their own specific characteristics. These differences may be due to these different genetic backgrounds or due to the subtle differences during cell culture. From the point of clinical application, it is necessary to build more cell lines; because if hESCs are used in clinical practice, the problem of immunological rejection

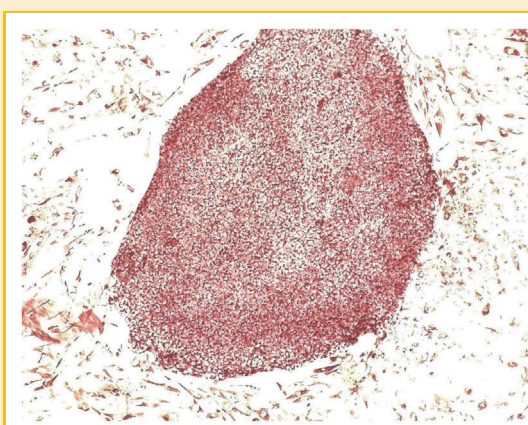

Fig. 2. AKP staining for hESC clones (20×, ZZU-hES-1, Passage 30) hESCs are fixed with paraformaldehyde, and then washed with PBS followed by addition of AKP for coloration. AKP-positive hESCs are violet-blue (strongly positive), while the feeder layer fails to be stained.

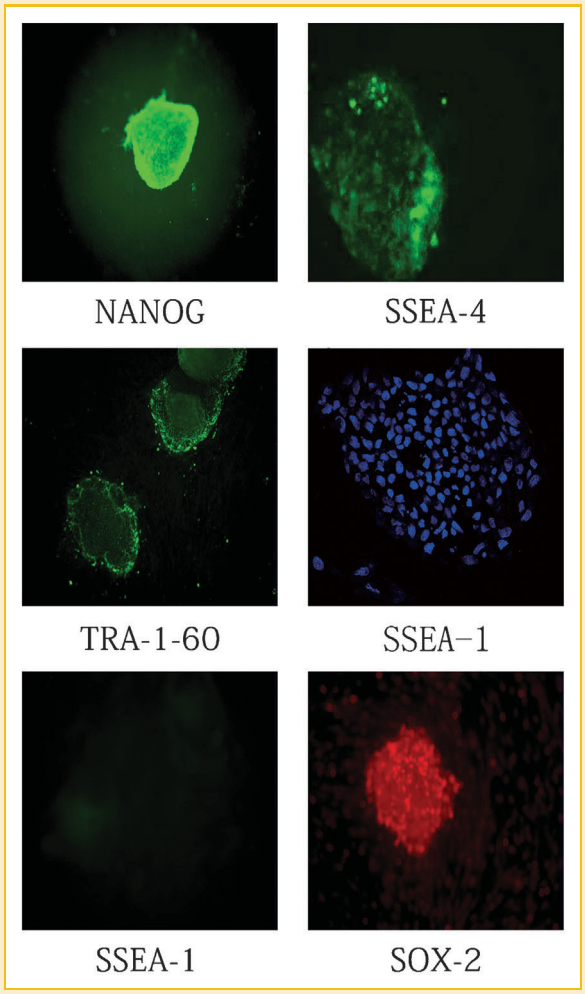

Fig. 3. Immunofluorescence for hESC clones (100×, ZZU-hES-5, Passage 36). After hESCs are fixed with paraformaldehyde, 1:40 (v: v) of SSEA-4, SSEA-1, NANOG, SOX2, and TRA-1-60 as first antibody are added for incubation at 4°C overnight. The next day, the corresponding secondary antibodies are added for incubation at 37°C for 30 min followed by observation under the fluorescence microscope. NANOG-positive hESC is green; SSEA-4-positive hESC is green; TRA-1-60-positive hESCs are green; SSEA-1 staining is negative in hESCs and SOX-2-positive hESC is red.

must be solved. However, doing HLA matching between donor and recipient cells is one of the methods to solve immunological rejection, which requires a certain number of available cell lines. Therefore, it is very important to establish hESCs from multiple sources.

DIFFERENCES IN DEVELOPMENT POTENTIAL BETWEEN DIFFERENT EMBRYOS

In IVF/ICSI-ET cycles, there often are some abnormal fertilized eggs which developed into the embryos with 0PN, 1PN, or multi-pronuclei (≥3PN, mainly 3PN) and the cleavage-arrested embryos with 2PN. Feng and Hershlag [2003] found that in IVF cycles, 50% of fertilized eggs with 1PN contained Y chromosome, and developed into the embryos with 1PN after cell cleavage; cytogenetics indicated these embryos usually possessed diploid karyotype,

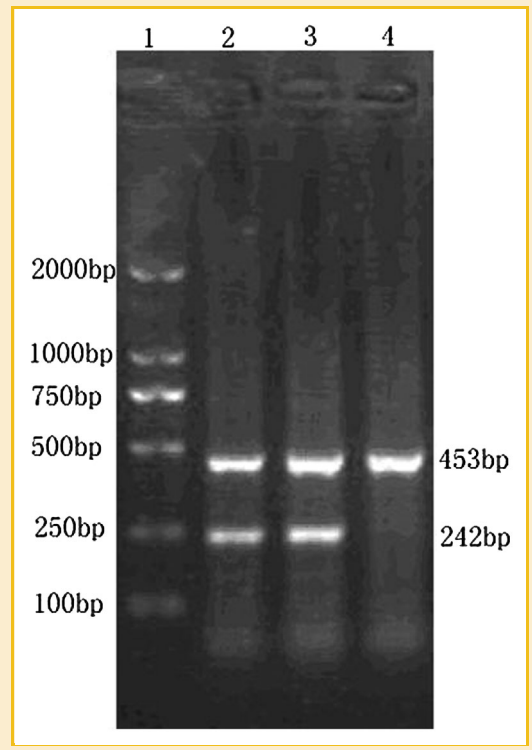

Fig. 4. OCT-4 expression in hESCs detected with RT-PCR (ZZU-hES-9, Passage 40 and ZZU-hES-13, Passage 29). RNA is extracted from hESCs with Trizol to synthesize cDNA through reverse transcription. After amplification, OCT-4 positive expression is obtained. Lane 1: Marker; Lane 2 and 3: OCT-4; Lane 4: β-actin.

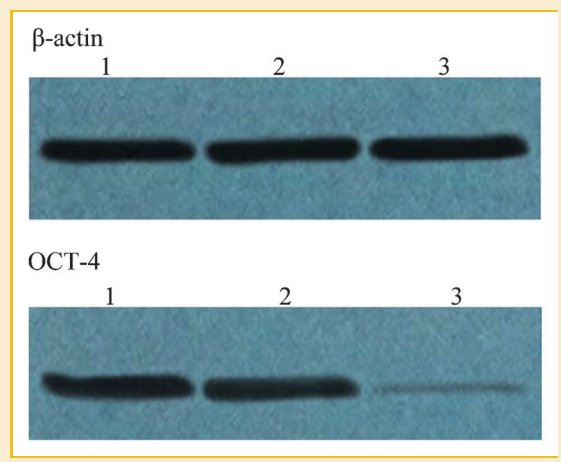

Fig. 5. OCT-4 expression in hESCs detected with Western blot (ZZU-hES-9, Passage 40 and ZZU-hES-13, Passage 29). After extracted from hESCs, the protein undergoes SDS-PAGE electrophoresis, and then is transferred to a nitrocellulose membrane. The membrane is sealed with TBST containing 10% of non-fat dry milk for 30 min followed by addition of rabbit anti-human OCT-4 antibody. After HRP-conjugated mouse anti-rabbit secondary antibody is added for 2-h incubation, the membrane undergoes exposure, visualization, and fixing followed by observation. Lanes 1 and 2: OCT-4; Lane 3: blank control.

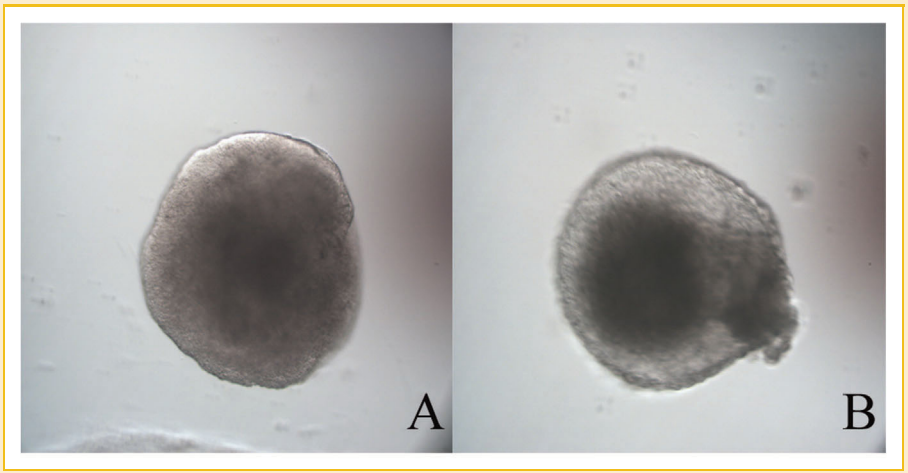

Fig. 6. hESCs in vitro differentiate into simple embryoid body (A) and cyst-shaped embryoid body (B) (20×, ZZU-hES-6, Passage 28). Cells are digested with trypsin, and then blown into single cell suspension. Simple embryoid body is formed 3 days after cell culture. After the simple embryoid body continues to be incubated for 5–7 days, capsular space occurs with the forming of cyst-shaped embryoid body.

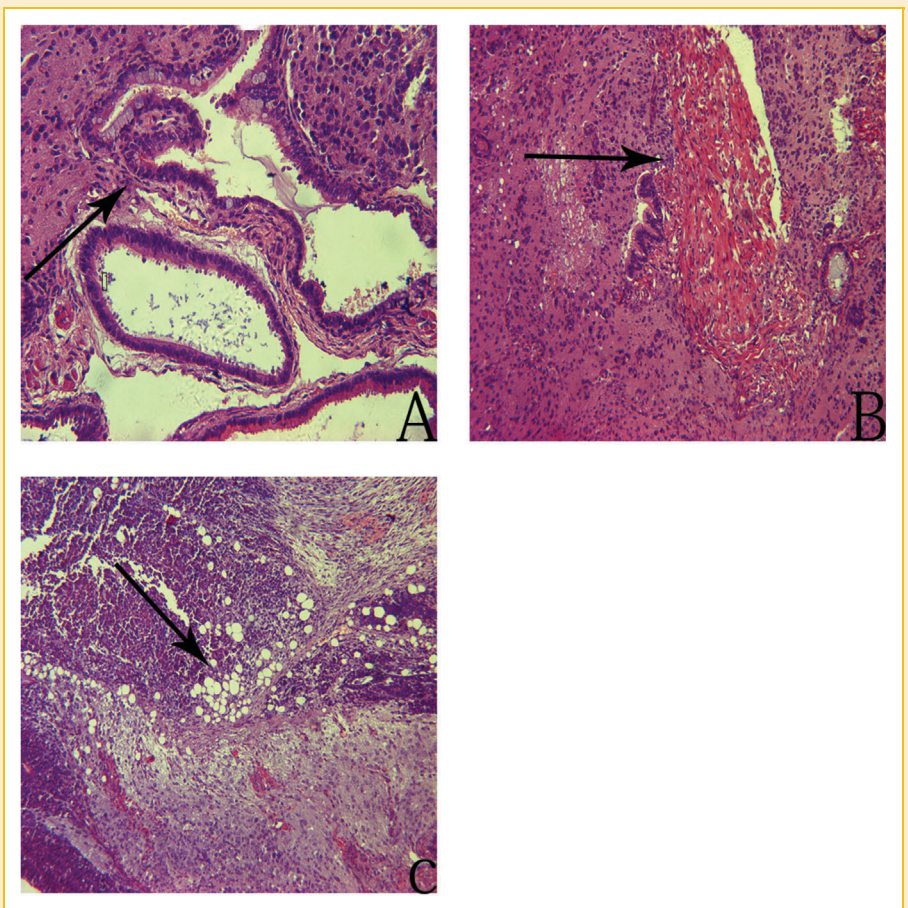

Fig. 7. hESCs differentiate into teratomas in vivo (20×, ZZU-hES-7, Passage 31). hESCs are mechanically cut into 50–100 small cell aggregates which are inoculated into SCID mice. Eight to 12 weeks later, teratomas are formed. The arrows indicate glandular tissue (A), muscles (B), and fat cells (C) in the immunohistochemical section.

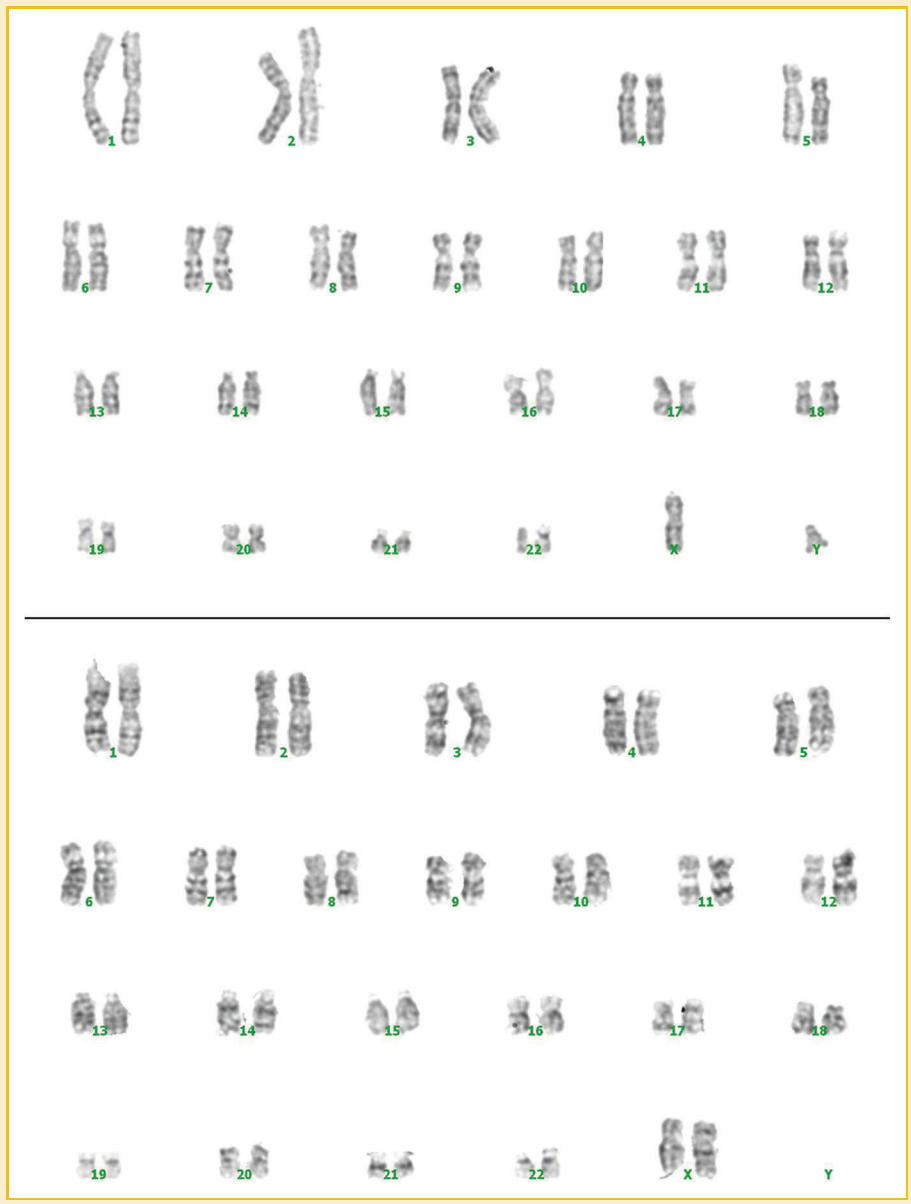

Fig. 8. Chromosome G-banding karyotype analysis of hESCs: 46, XY (ZZU-hESC-2, passage 29) and 46, XX (ZZU-hESC-3, passage 20). hESCs are incubated with colchicine, and then digested with trypsin. Samples are fixed with KCl, and stained with Giemsa solution followed by observation under oil immersion lens.

suggesting they belonged to normal fertilization. Our results showed that the blastulation rate was the highest in the embryos with 1PN, the second in the embryos with 0PN, and lower in the embryos with 3PN and the cleavage-arrested embryos with 2PN. This suggests that in discarded embryos, embryos with 0PN or 1PN have better developmental potential. In clinical practice, if there is no normal 2PN fertilized eggs, the embryos with 0PN or 1PN are selected to use in embryo transfer; but it is necessary to carry out chromosomal screening with the techniques of pre-implantation genetic diagnosis and prenatal diagnosis before embryo implantation. It can be seen from our results of large-scale discarded embryos that the rates of normal chromosomes and hESC establishment are high in the embryos with 0PN or 1PN. Our results are consistent with the above principle of embryo transfer, and verify the rationality of this principle to select abnormal fertilized embryos for embryo transfer.

EMBRYO QUALITY IS CLOSELY RELATED TO BLASTOCYST FORMATION

Many studies indicate that low-quality embryos have an adverse effect on blastulation rate, blastocyst quality, implantation rate, and live birth rate. Alikani et al. [2000] studied the relations between embryo morphology in cleavage-stage and normal blastocyst formation; and found that cleavage speed is associated with blastocyst formation, the blastulation rate was 41.9% in D_3 embryos with 7–9 blastomeres, while in the embryos with <7 or >9 blastomeres the blastulation rates were significantly decreased. Neuber et al. [2003] have described that high-quality D_3 embryos

with $\geq$7 blastomeres more readily developed into high-quality blastocysts; while for poor-quality embryos, it was difficult to develop into high quality blastocysts. Racowsky et al. [2000] found that the live birth rate was only 3% in D_3 embryos with $<$7 blastomeres, but it was 18% and 25% in D_3 embryos with 7 or 8 blastomeres, respectively. In our study, the blastulation rate is high in D_3 embryos with 7–9 blastomeres, which provides a reference for clinical blastocyst culture.

RELATION BETWEEN HIGH-QUALITY BLASTOCYSTS AND hESC ESTABLISHMENT

In vitro culture of hESCs must be under the conditions both to promote hESC proliferation and to retain undifferentiated diploid state which can differentiate into three germ layers-derived cells. In vitro culture of hESCs is associated with blastocysts, feeder layer, culture media, growth factors, etc. High-quality blastocysts are important for hESC establishment.

Intact ICM is easily obtained from high-quality blastocysts because cell number is more and cell proliferation is strong in high-quality blastocysts; in contrast, ICM is easily scattered, dissolved and lost during separating ICM from low-quality blastocysts. Therefore, high-quality blastocysts can improve hESC establishment rate. In this study, the hESC establishment rate of high-quality blastocysts was 19.42% and low-quality blastocysts was 0.70%. The hESC establishment rate of normal embryos is 3–10% [Hanson and Caisander, 2005].

SIGNIFICANCE OF DISCARDED EMBRYO-DERIVED hESC ESTABLISHMENT

Embryonic stem cells possess unlimited proliferative capacity and can differentiate into three germ layers-derived cells in vitro under specific conditions, so they are the best source of transplant donor in regenerative medicine. Immunological rejection still is a main problem of cell transplantation, one of the strategies to overcome immunological rejection is to establish a human embryonic stem cell bank with a variety of human histocompatibility antigens. However, ethical issues and embryo sources still affect hESC establishment.

At present, most hESCs come from the remanent embryos after assisted reproductive treatment cycles. Gavrilov et al. [2011] successfully established two hESCs with discarded retarded D_5 blastocysts. Suss-Toby et al. [2004] established a hESC line with normal karyotype using 1PN embryos. In addition, with the development of assisted reproductive technology (ART), there are a lot of cryopreserved embryos in the world. Some couples have successfully got children by ART, it is not necessary to store their frozen embryos. Therefore, many scholars believe that under the couples' consent, the remaining frozen embryos may be used for scientific research [Heng, 2006]. At present, the use of clinical discarded fresh or frozen embryos can solve the problems including the lack of embryos and ethic disputes in the establishment of human embryonic stem cell bank.

In our Reproductive Center, about 2,000 IVF cycles are performed every year, and there are a large number of discarded fresh or frozen embryos which provide a abundant embryo resources for the establishment of human embryonic stem cell bank. In this study, a total of 22 hESCs have been established, and are all consistent with the current identification criteria of hESCs. The identification results indicate that the hESCs we established all have normal karyotype, which may be that it is difficult to successfully establish the hESCs with abnormal karyotype because they have been eliminated during passages. When we use discarded fresh embryos to establish hESC lines, D_3 0PN or 1PN embryos with 7–9 blastomeres should be first selected in order to improve high-quality blastulation rate which can increase the efficiency of hESC establishment. This study has important significance for establishing hESC lines with discarded embryos.

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