

Human embryonic stem cell lines derived from the Chinese population

Zhen Fu FANG^{1,2}, Fan JIN³, Hui GAI¹, Ying CHEN¹, Li WU¹, Ai Lian LIU¹, Bin CHEN¹, Hui Zhen SHENG^{1,2,*}

¹Center for Developmental Biology, Xinhua Hospital, Shanghai Second Medical University, 1665 Kong Jiang Road, Shanghai 200092, China

²Laboratory of Stem Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

³In vitro Fertilization Center of Women's Hospital, School of Medicine, Zhejiang University, Zhejiang 310006, China

ABSTRACT

Six human embryonic stem cell lines were established from surplus blastocysts. The cell lines expressed alkaline phosphatase and molecules typical of primate embryonic stem cells, including *Oct-4*, *Nanog*, *TDGF1*, *Sox2*, *EBAF*, *Thy-1*, *FGF4*, *Rex-1*, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81. Five of the six lines formed embryoid bodies that expressed markers of a variety of cell types; four of them formed teratomas with tissue types representative of all three embryonic germ layers. These human embryonic stem cells are capable of producing clones of undifferentiated morphology, and one of them was propagated to become a subline. Human embryonic stem cell lines from the Chinese population should facilitate stem cell research and may be valuable in studies of population genetics and ecology.

Keywords: human embryonic stem cell, pluripotent, cell line, teratoma.

INTRODUCTION

Since first reported in 1998 [1], additional human embryonic stem (hES) cell lines have been established [2-5]. Although all hES cell lines can be propagated in an undifferentiated state, they vary significantly in other biological properties such as proliferation speed, cloning efficiency and tendency to differentiate spontaneously in culture [1,6,7]. Thus, it is strongly recommended that more hES cell lines should be derived and studied, in order to better understand the mechanisms of variability and to screen cell lines suitable for various applications in regenerative medicine [8]. For these reasons, six hES cell lines were established from the Chinese population and characterized in a comparative way.

MATERIALS AND METHODS

hES cell culture

Human embryos were produced for clinical purposes using an *in vitro* fertilization (IVF) procedure. Surplus embryos were used to derive hES cells. The protocols used for IVF and for hES cell deriva-

tion were in accordance with the stem cell guidelines issued by the Ministry of Science and Technology and the Ministry of Health, and were approved by the Ethical Committee of Xinhua Hospital.

From each blastocyst used, the inner cell mass (ICM) was isolated manually and cultured on irradiated mouse embryonic fibroblasts (MEF) at a density of 25,000 cells/cm². The culture medium consisted of 78% DMEM/F-12, 20% KnockOut serum replacement (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 1% non-essential amino acids, 0.1 mM β -mercaptoethanol, and 4-8 ng/ml human recombinant bFGF. Ten to fourteen days after initial plating, the outgrowth of the ICM was transferred to a dish with fresh feeders. Passaging of hES cells was performed by a combination of enzyme digestion (0.05% trypsin) and manual dissociation [9]. hES cells were cryo-preserved in media consisting of 45% KnockOut serum replacement, 45% DMEM/F12 and 10% DMSO.

To determine the population doubling (PD) time, small sized colonies were marked, photographed once every 24 h, and the cell number in each colony was counted. PD values were calculated by the following formula: PD (h) = total time/Log₂(N₁/N₂).

Cell cloning

hES colonies were dissociated into single cell using 0.05% trypsin. A total of 1×10^6 cells per cell line were seeded in the culture dish. Ten days later, the colonies were counted and their cloning efficiencies were calculated with the following formula: cloning efficiency = (number of colonies/100,000) \times 100. To derive subclones from a cell line, individual cells were manually picked and placed in a 96-well culture plate.

*Correspondence: Hui Zhen SHENG
Tel/Fax: 86-21-55570017
E-mail: hzsheng@sh163a.sta.net.cn

Tab. 1 Primers and PCR products for Class I gene products

Gene	Accession No.	Primer sequence	Position	Product size (bp)
<i>Oct-4</i>	NM_002701.1	S 5' TGGGGGTTCTATTGGGAAGG 3' A 5' GTTCGCTTCTCTTCGGGC 3'	328-520	193
<i>Nanog</i>	NM_024865	S 5' TGATTCTTCCACCAGTCC 3' A 5' TGGAGGCTGAGGTATTTC 3'	414-584	171
<i>TDGF1</i>	NM_003212	S 5' ATGGCCCCGTTCTCTTAC 3' A 5' GTTCCGTCCGTAGAAGGAG 3'	263-562	300
<i>Sox2</i>	NM_003106	S 5' CAACGGCAGCTACAGCA 3' A 5' GGAGTGGGAGGAAGAGGT 3'	928-1210	283
<i>EBAF</i>	NM_003240	S 5' TGTCTTCCCGTCCATCA 3' A 5' AGCCCTTCATCCTTCCTC 3'	1325-1670	346
<i>Thy-1</i>	NM_006288	S 5' CCCCAGCAATCCCTCAAAC 3' A 5' CCCAATCCTGGCTTCCCT 3'	622-848	227
<i>FGF4</i>	NM_002007	S 5' GGGCGTGGTGAGCATCTT 3' A 5' CTTCTTGGTCTTCCCATTCTTG 3'	688-886	199
<i>Rex1</i>	AF450454	S 5' GCGTACGCAAATTAAGTCCAGA 3' A 5' CAGCATCCTAAACAGCTCGCAGAAT 3'	488-793	306
<i>β-actin</i>	BC013835	S 5' CCACGAAACTACCTTCAACTC 3' A 5' GCCATGCCAATCTCATCTT 3'	894-1280	387

in vitro differentiation

hES cells were harvested as large clumps and cultured in 78% DMEM, 20% FBS, 2 mM L-glutamine, 1% non-essential amino acids, and 0.1 mM β-mercaptoethanol, in a non-adherent Petri dish for 4-6 d to form embryoid bodies (EBs) [10]. For further differentiation, EBs were transferred to gelatin coated culture dishes. After approximately 3 wks, a variety of morphologically distinct cells grew from the EBs. Total RNA extracted from these cells was analyzed for the expression of *Neurofilament*, *Nestin*, (ectoderm), *Bone morphologic protein 4 (BMP4)*, *Myoglobin*, (mesoderm), *α-fetoprotein* and *Albumin* (endoderm) using the following primer sets:

Neurofilament: 5'-ACCCGACTCAGTTTCACCA, 5'-TTCTT CACCTTCACCTCCTTC

Nestin: 5'-GAGGACCAGAGTATTGTGAGAC, 5'-CACAGT GGTGCTTGAGTTTC

BMP4: 5'-TTTGTTC AAGATTGGCTGTC, 5'-AGATCCCGC ATGTAGTCC

Myoglobin: 5'-GCCACCAAGCACAAGATC, 5'-CTGCCAC CTCTACTAAACA

α-fetoprotein: 5'-CTTTGGGCTGCTCGCTATGA, 5'-TGGCT TGGAAAGTTCGGGTC

Albumin: 5'-ATGCGCTATTAGTTCGTTA, 5'-TGTCACCT ACTGGCGTTTT

Teratoma formation

Approximately 2-5×10⁶ morphologically undifferentiated hES cells were injected into the thigh muscles of 4-w-old NOD-SCID mice (from Shanghai Laboratory Animal Center, Chinese Academy of Sciences). After tumor formation, which was usually a period of 10 to 12 wks, the teratomas were dissected, fixed in 4% paraformaldehyde and examined histologically after hematoxylin and eosin staining.

RT-PCR

Total RNA was extracted from hES cells using Trizol® (Invitrogen, Carlsbad, CA) and then treated with DNase I at 37°C for 20 min. First strand synthesis was performed using Moloney Murine Leukemia Virus reverse transcriptase (Promega, Madison, WI) and random Primers (Promega) according to the manufacturer's instructions. Primer sequences are listed in Tab. 1. PCR reactions were conducted using the following parameters: denaturation at 94°C for 4 min followed by 35 amplification cycles, denaturation at 94°C for 30 s, annealing for 30 s, and extension at 72°C for 40 s. The cycles were followed by a final extension at 72°C for 5 min. Annealing temperatures were as follows: 55°C for *Oct-4*, *TDGF1*, *Thy-1*, *FGF4*; 60°C for *Nanog*, *Sox2*, *EBAF*; and 65°C for *Rex1*. Samples without reverse transcriptase, at the reverse transcription step, were used as the control for genomic DNA contamination. *β-actin* was used as an internal standard.

Immunocytochemistry

The immunocytochemical procedure was performed using protocols recommended by the manufacturer of the reagents. In brief, after washing with phosphate buffered saline (PBS), hES cell colonies were fixed in 4% paraformaldehyde for 15 min at room temperature (RT). Colonies were washed twice (5-10 min each) in TBST buffer (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween-20), treated with 0.1% Triton X-100/PBS for 10 min, washed twice (5-10 min each) in TBST buffer and incubated in 3% BSA/PBS (blocking solution) for 1 h. The primary antibodies used were against SSEA-1, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 from Chemicon (Temecula, CA) and OCT-4 from Santa Cruz Biotechnology (Santa Cruz, CA). All antibodies were diluted in PBS and incubated with samples overnight at 4°C. Next day, colonies were washed thrice with TBST buffer and incubated with secondary antibodies at RT for

1 h. The secondary antibodies used were FITC-conjugated donkey anti-goat IgG, FITC-conjugated goat anti-mouse IgG + IgM, FITC-conjugated donkey anti-mouse IgG, and FITC-conjugated goat anti-rat IgG + IgM (Jackson, West Grove, PA). Fluorescent signals were visualized with a confocal microscope (Olympus).

To detect alkaline phosphatase activity, hES cell colonies were fixed in 4% paraformaldehyde for 10 min at RT, rinsed in a Tris buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂, pH9.5), and reacted with NBT/BCIP in darkness for approximately 15 min. The reaction was stopped when the color had developed sufficiently.

Karyotyping

hES cells were incubated with colcemid (40 µg/ml) for 3 h at 37°C 5% CO₂. Cells were trypsinized and pelleted at 1,200 rpm for 5 min in an eppendorf centrifuge (5810 R), resuspended in 4 ml of hypotonic solution (KCl 0.56 g/100 ml H₂O + sodium citrate 0.5 g/100 ml H₂O), incubated at 37°C for 30 min, mixed with 2 ml of fixative (acetic acid:methanol = 1:3, added dropwise) and incubated at RT for 5 min. Cells were centrifuged at 1,200 rpm for 5 min, and then resuspended in 2 ml of fixer. The centrifugation and fixing steps were repeated three times. On the last repeat, cells were kept in the fixative and dropped onto pre-chilled glass slides. Chromosome spreads were placed at RT for 2 d, baked at 65°C for 4 h, digested with 0.1% trypsin (in 0.8% NaCl) at 37°C for 5 min, stained with 1% Giemsa solution for 15 min and photographed. At least 20 metaphase spreads were counted and five Giemsa banded karyotypes were analyzed for each hES cell line.

HLA type analysis

The HLA allele was determined using PEL-FREEZ ABC SSP UNITRAY II kits and PEL-FREEZ DR/DQ3T SSP UNITRAY kits (PEL-FREEZ® Biotechnology, Beijing, China), according to the manufacturer's instructions.

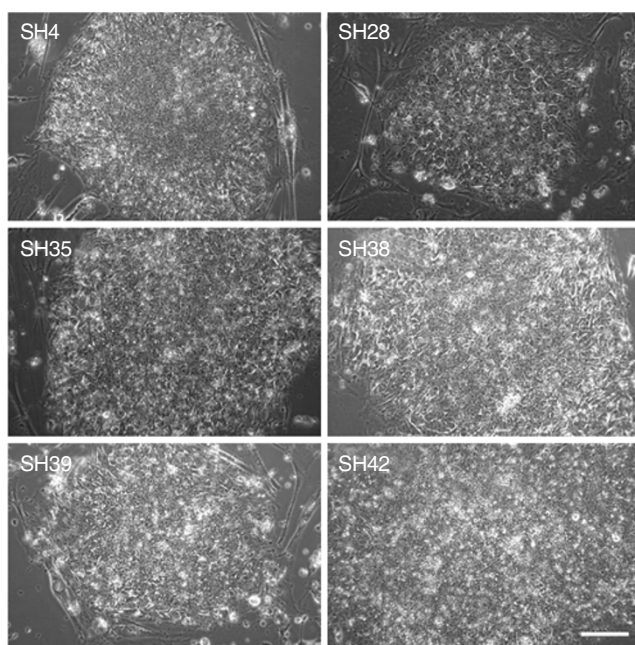


Fig. 1 Morphology of colonies of six hES cell lines. Scale bar=100 µm.

RESULTS

Derivation and long term culture of hES cells

ICM was placed on MEF for approximately 2 wks. The expanded ICM was transferred to new dishes with fresh feeders. Six cell lines were established from a total of forty-two blastocysts. hES cells grew as a monolayer with a high nuclear-to-cytoplasm ratio and prominent nucleoli. At early passages, some lines grew as cell clusters without a defined border. However, in prolonged culture, all colonies from the six lines (SH4, SH28, SH35, SH38, SH39, SH42) developed clear edges (Fig. 1). PD values were calculated using the H1 line [1] as the control, which showed a PD time of approximately 30 h in our culture. The six newly established cell lines showed PD values that ranged from 30 to 39 h (Tab. 2). At present, SH4, SH28, SH35, SH38, SH39, SH42 have reached approximately 279, 268, 200, 210, 172, 198 PD, respectively. Karyotypes of the six cell lines, SH4 at PD 171, SH28 at PD 124, SH35 at PD 80, SH38 at PD 90, SH39 at PD 75, and SH42 at PD 80, were analyzed by Giemsa banding. All had normal karyotypes of 46, with SH4, SH28, SH35 and SH38 as XX, and SH39 and SH42 as XY (Fig. 2). These results demonstrate that the cell lines can be cultured for prolonged periods without compromising their chromosomal stability.

Marker expression by hES cell lines

The cell lines were analyzed for expression of Class I markers [11] using the RT-PCR method. As shown in Fig. 3, each of the lines examined expressed a full panel of class I transcripts, including *Oct-4*, *Nanog*, *TDGF1*, *Sox2*, *EBAF*, *Thy-1*, *FGF4*, and *Rex1*. Expression of OCT-4 and Class II antigens by the cell lines was examined using specific antibodies. As shown in Fig. 4, all six lines were positively labeled with antibodies against OCT-4, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81, but not with the

Tab. 2 Population doubling time and cloning efficiencies of six hES cell lines

Cell lines	PD time (h)	Cloning efficiency (%)
SH4	32±2	0.592±0.010
SH28	30±3	0.484±0.017
SH35	36±1	1.143±0.018
SH35a	33±4	N.D*
SH38	32±3	0.820±0.012
SH39	39±3	0.138±0.013
SH42	34±2	0.745±0.010
H1	30±2	0.413±0.006

* not done

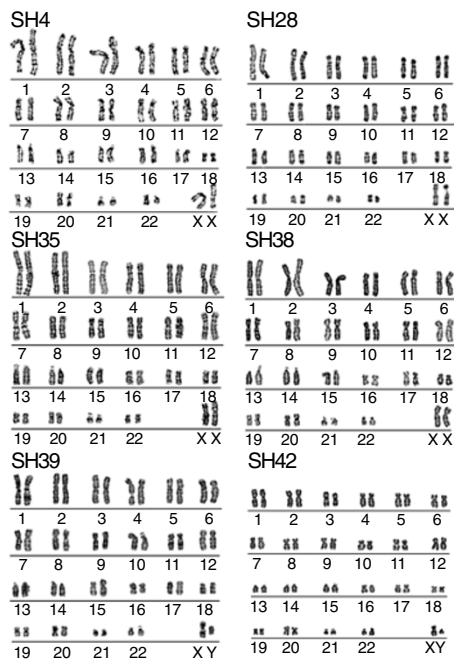


Fig. 2 Karyotype analysis of hES cell lines using the G-band method showing normal 46, XX karyotypes for the SH4, SH28, SH35, SH38 lines and normal 46, XY karyotypes for the SH39 and SH42 lines.

antibody against SSEA-1 (data not shown). These lines also expressed a high level of alkaline phosphatase activity (Fig. 4).

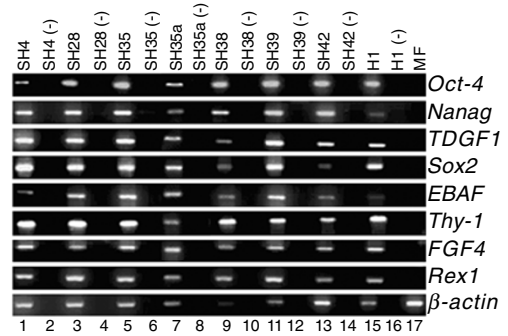


Fig. 3 Expression of *Oct-4*, *Nanog*, *TDGF1*, *Sox2*, *EBAF*, *Thy-1*, *FGF4*, *Rex1* in six hES cell lines and one subline (lanes 1, 3, 5, 7, 9, 11 and 13). Negative controls were DNA samples from each line that underwent the same protocol as the experimental group, except that reverse transcriptase was not added during reverse transcription (lanes 2, 4, 6, 8, 10, 12, 14 and 16). Lane 15, expression of Class I genes by the H1 line. Lane 17, mouse feeder (MF). β -actin transcripts were detected in each of the samples.

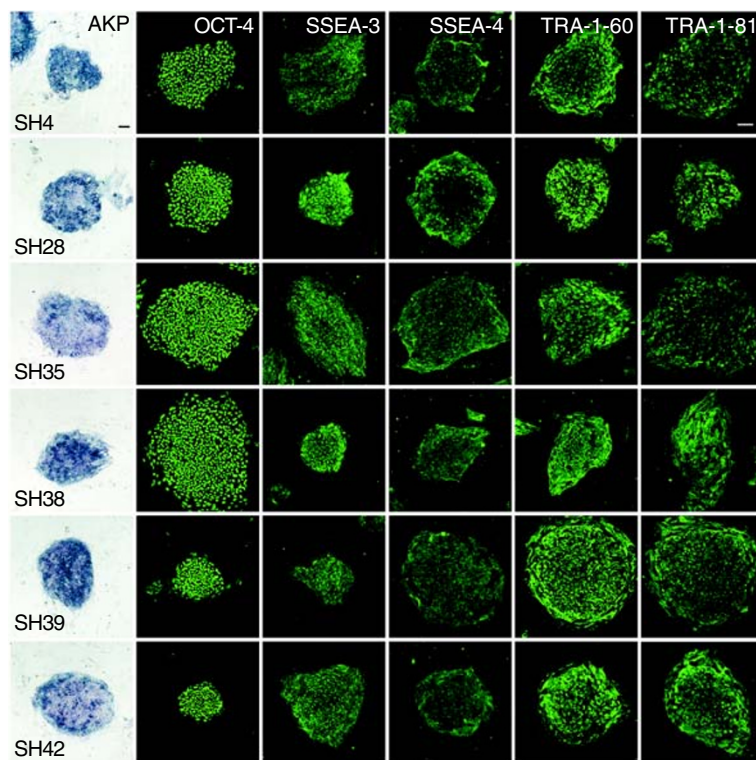


Fig. 4 Photomicrographs shows alkaline phosphatase activity (AKP) and the expression of OCT-4, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 in the six cell lines. Scale bars=100 μ m.

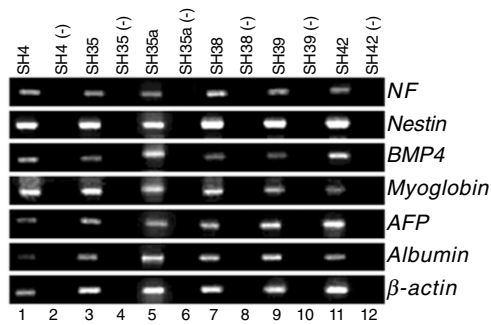


Fig. 5 Results from the reverse transcription PCR assay. Differentiated cells from the hES lines expressed genes representative of three embryonic germ layers (lanes 1, 3, 5, 7, 9 and 11). Lanes 2, 4, 6, 8, 10 and 12 were negative controls. β -actin was used as an internal standard. *NF*, *Neurofilament*; *Nestin* (ectoderm); *BMP4*, *Bone morphogenesis protein*; *Myoglobin* (mesoderm); *AFP*, α -fetoprotein; *Albumin* (endoderm).

Cloning efficiency and derivation of a cloned subline

The cloning efficiencies of the hES cell lines were evaluated using the H1 cell line as a control. hES cell colonies were dissociated into individual cells using a trypsin digestion (0.05%, 37°C for 8 min) and plated on MEF in the standard medium for hES cells. In our culture, the H1 line gave a cloning efficiency of 0.413%. All six newly established cell lines yielded undifferentiated cell colonies. Four lines showed cloning efficiencies close to the H1 line, one line showed a particularly low cloning efficiency (SH39, 0.138%), and one line showed a particularly high cloning efficiency (SH35, 1.143%; see Tab. 2).

A cloned subline (SH35a) was established from the SH35 line. The SH35a line had a PD value of 33 h. It formed EBs and differentiated into a variety of cell types, in a manner similar to the parent line (Fig. 5). These results demonstrate that the SH35 line is capable of generating equipotent subclones.

Differentiation *in vitro*

EBs, which are cellular aggregates with round and smooth surfaces, were formed from all of the hES cell lines (Fig. 6A, C-F), with the exception of the SH28 line. For further differentiation, EBs from five hES cell lines were plated onto the surface of the culture dish. Differentiated cells grown out of the EBs were highly heterogeneous in morphology (Fig. 6B). Marker analyses with primers specific for *Neurofilament*, *Nestin* (ectoderm), *BMP4*, *Myoglobin* (mesoderm), α -fetoprotein and *Albumin* (endoderm) showed that differentiated cells from the five hES cell lines expressed markers representative of all three germ layers (Fig. 5) [12]. In contrast to other hES cell

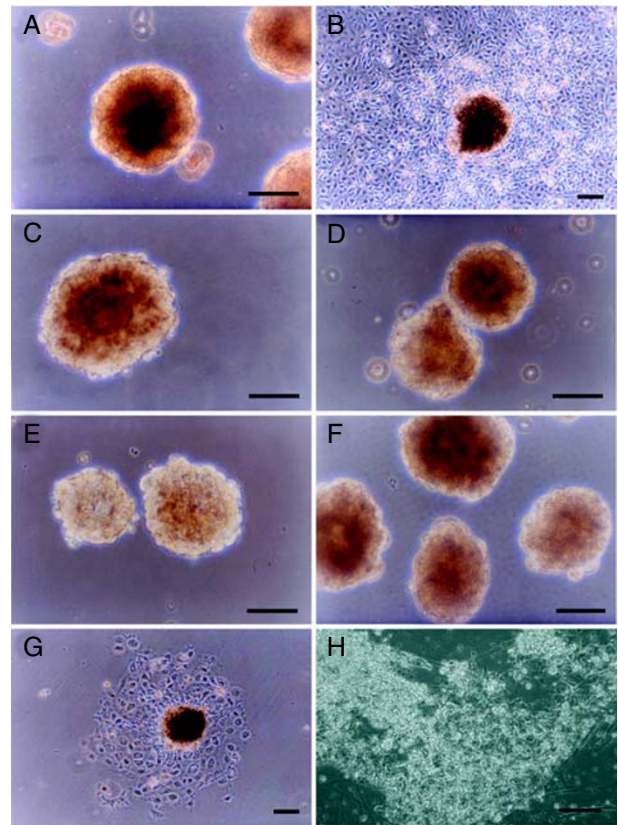


Fig. 6 Micrographs showing EB formation (A, C-F), differentiated cells grown out of the EB (B, G) and SH28 cultured in high-density (H). (A, B) SH35; (C) SH4; (D) SH38; (E) SH39, (F) SH42, (G, H) SH28. Scale bars=100 μ m.

lines, cell clumps from the SH28 line remained as clumps with irregular surfaces. Only a few SH28 cell clumps attached to the surface of the culture dish. In these clumps, we observed much less outgrowths of differentiated cells in comparison with other 5 cell lines (Fig. 6G). In high density culture, cell clumps from the SH28 line lost cells, while those at the bottom of the dish remained as a monolayer with morphological characteristics similar to undifferentiated hES cells (Fig. 6H).

Differentiation *in vivo*

The differentiation potentials of the six lines were further evaluated by observing teratoma formation. Dissociated hES cells were injected into the thigh muscles of NOD-SCID mice. Teratomas developed from cells of the SH35, SH38, SH39 and SH42 lines. These teratomas contained multi-differentiated tissues, such as keratinized stratified squamous epithelium, neural epithelium, pigment epithelium (ectoderm), bone, cartilage (mesoderm) and gut epithelium, as well as pseudostratified ciliated columnar

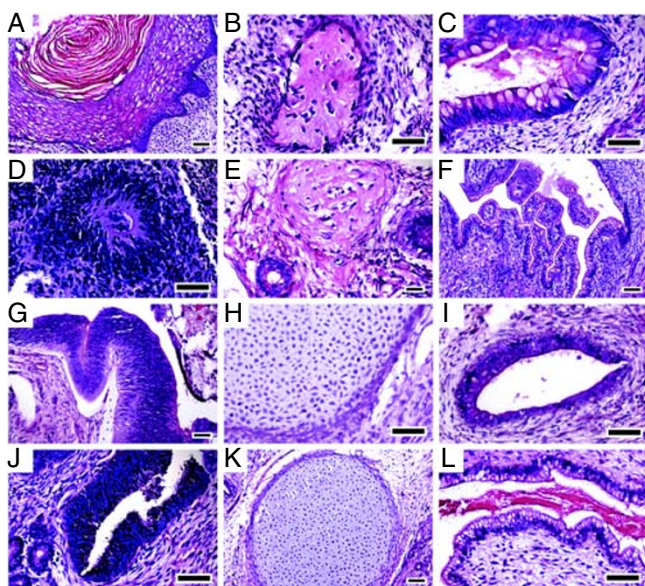


Fig. 7 Histological analysis of teratomas. (A-C) SH35; (D-F) SH38; (G-I) SH39; (J-L) SH42. Teratomas contained multi-differentiated tissues. Ectoderm (A, D, G, J): keratinized stratified squamous epithelium (SH35), neural epithelium (SH39), and pigment epithelium (SH38, SH42). Mesoderm (B, E, H, K): bone (SH35, SH38) and cartilage (SH39, SH42). Endoderm (C, F, I, L): gut epithelium (SH35, SH38) and pseudostratified ciliated columnar epithelium (SH39, SH42). Scale bars=50 μ m.

Tab. 3 HLA isotype of six hES cell lines

Cell line	HLA-A	HLA-B	HLA-C	HLA-DRB1	HLA-DQB1
H4	02/11	46/48	01/08	1125/09	03/03
H28	02/33	40/58	03/07	03/08	06/02
H35	02/11	27/46	01/15	15/11	06/03
H38	02/02	46/51	01/15	15/13	06/06
H39	24/11	52/58	03/1202	08/09	03/04
H42	02/11	40/58	03/07	03/04	02/04

epithelium (endoderm; see Fig. 7). The SH4 and SH28 lines did not produce teratomas, despite repeated injections into the thigh muscles.

HLA typing

Compatibility between the donor and recipient HLA genes is a critical factor in the success of cell or organ transplantation [13]. The HLA type of a cell line is also important for the interpretation of experimental results related to immune responses. To facilitate future research using hES cells, we determined the HLA types of the six cell lines and presented the results in Tab. 3.

DISCUSSION

We established six hES cell lines from the Chinese population. All six cell lines had morphological characteristics typical of hES cells, and all six lines expressed class I and class II markers that are characteristic of hES cells. These lines were propagated for many generations in culture; the lines remained undifferentiated, and retained normal karyotypes. Five of the six lines (SH4, SH35, SH38, SH39, and SH42) were capable of forming EBs and four of them (SH35, SH38, SH39, and SH42) formed teratomas.

As previously reported for other cell lines [14], we observed variations among the six cell lines in proliferation speed, cloning efficiency, and the capacity to form teratomas. It is surprising that cells of the SH28 line expressed most of the markers of undifferentiated hES cells, but differentiated poorly. The reason for this observation needs to be further investigated.

Variability among hES cell lines may arise from properties of the founder cells as well as differences in culture conditions. hES cells are derived from the ICM of blastocysts, which consists of a transient cell population in a continuous process of differentiation into epiblasts and then into three primary germ layers [15]. The procedure used to isolate hES cells is, in essence, a process of selection for cells that have acquired the ability to proliferate in response to the culture environment. Therefore, cell line characteristics may reflect the differentiation state of its founder cell(s). As more hES cell lines are established and analyzed, we may obtain additional detailed information regarding the differentiation process of the ICM.

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REFERENCES

- 1 Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. *Science* 1998; **282**:1145-7.
- 2 Stem Cell Information. National Institutes of Health. <http://stemcells.nih.gov/research/registry/>
- 3 Hovatta O, Mikkola M, Gertow K, et al. A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. *Hum Reprod* 2003; **18**:1404-9.
- 4 Zeng X, Miura T, Luo Y, et al. Properties of pluripotent human embryonic stem cells BG01 and BG02. *Stem Cells* 2004; **22**: 292-312.
- 5 Suss-Toby E, Gerecht-Nir S, Amit M, Manor D, Itskovitz-Eldor J. Derivation of a diploid human embryonic stem cell line from a mononuclear zygote. *Hum Reprod* 2004; **19**:670 - 5.
- 6 Cowan CA, Klimanskaya I, McMahon J, et al. Derivation of embryonic stem-cell lines from human blastocysts. *N Engl J Med* 2004; **350**:1353-6.
- 7 Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro*. *Nat Biotechnol* 2000; **18**:399-404.
- 8 Gearhart J. New human embryonic stem-cell lines - more is better. *N Engl J Med* 2004; **350**:1275-6.
- 9 Hwang WS, Ryu YJ, Park JH, et al. Evidence of a pluripotent human embryonic stem cell line derived from a cloned blastocyst. *Science* 2004; **303**:1669-74.
- 10 Amit M, Shariki C, Margulets V, Itskovitz-Eldor J. Feeder layer- and serum-free culture of human embryonic stem cells. *Biol Reprod* 2004; **70**:837-45.
- 11 Brivanlou AH, Gage FH, Jaenisch R, et al. Setting standards for human embryonic stem cells. *Science* 2003; **300**:913-6.
- 12 Itskovitz-Eldor J, Schuldiner M, Karsenti D, et al. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol Med* 2000; **6**: 88-95.
- 13 Drukker M, Benvenisty N. The immunogenicity of human embryonic stem-derived cells. *Trends Biotechnol* 2004; **22**:136-41.
- 14 Heins N, Englund MC, Sjoblom C, et al. Derivation, characterization, and differentiation of human embryonic stem cells. *Stem Cells* 2004; **22**:367-76.
- 15 Smith A. Embryonic stem cells. In: Marshak DR, Gardner RL, Gottlieb D, eds. *Stem cell biology*. Cold Spring Harbor Laboratory Press: New York 2001:205.