Chinese Science Bulletin

Maintenance of human embryonic stem cells on gelatin

LI Yang[1*], LIN ChangSheng[1*], WANG Li[1*], LIU Ying[1,2], MU XiaoNing[3], MA Yue[3] & LI LingSong[1†]

[1] Peking University Stem Cell Research Center and China National Center for International Research, Beijing 100191, China;
[2] Department of Reproduction, Beijing Obstetrics and Genecology Hospital, Beijing 100026, China;
[3] The Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

Matrigel is routinely used as a coating material in the feeder-free culture system of human embryonic stem cells (hESCs). However, matrigel is costive and inconvenient to use. In this study, the possibility of using gelatin as an alternative coating material was investigated. The results showed that, after trypsinization, hESCs were maintained undifferentiated on gelatin. These hESCs expressed pluripotent markers, formed teratoma and maintained a normal karyotype. As measured at passage 10, the hESCs expressed a high level of Oct4 on both gelatin and Matrigel. hESCs growing on gelatin formed AP-positive colonies in similar size and number to those growing on Matrigel ($P > 0.05$). Moreover, hESCs growing on gelatin contained a comparable percentage of SSEA-4-positive cells to those growing on Matrigel (95.1% vs.94.3%, $P > 0.05$). H-1 hESCs were maintained undifferentiated on gelatin for 20 passages and remained the stable normal karyotype. This gelatin-based culture protocol may allow us to propagate hESCs in large scale, with less cost.

human embryonic stem cells, gelatin, Matrigel, feeder-free, pluripotency

Human embryonic stem cells (hESCs) have a great potential for therapeutic treatment of certain human diseases[1,2]. A cost-effective and easily-handling culture system for expanding hESCs while retaining their pluripotency is crucial for regenerative therapies[3]. It is important to find a lower cost coating matrix as an alternative to Matrigel because hESCs are anchorage-dependent when growing as colonies[4–8].

With appropriate media supplemented with high concentrations of bFGF, several types of matrix have been demonstrated to support undifferentiated growth of hESCs with varying efficacy[4–7]. Laminin and Matrigel were able to maintain the growth well, while collagen IV failed to do so[4,7–11]. If grown on fibronectin, hESCs differentiate soon after replacing the cell[5,8,11]. Other alternative substrates, including human serum[12], MEF-derived extracellular matrix[7,10] and synthetic materials[13,14], have also been tested for supporting undifferentiated growth of hESCs.

Gelatin is able to support undifferentiated growth of mouse embryonic stem cells, and is more cost effective than most of the materials mentioned above. However, it has been noted that dissociated by collagenase IV, gelatin failed to support undifferentiated growth of hESCs[11]. Since gelatin contains substrates of Collagenase IV, we hypothesize that this failure may be due to the use of collagenase IV.

To test the above hypothesis, in the present study, we investigated whether gelatin could support the long-term propagation of hESCs if dissociated by trypsin instead of collagenase IV.

1 Methods and materials

1.1 Establishment and culture of the hESC line (PUSC-1).

Human embryos were obtained for clinical purposes by

Received June 29, 2009; accepted August 26, 2009
doi: 10.1007/s11434-009-0630-4
†Corresponding author (email: lingsongli@bjmu.edu.cn)
* Contributed equally to this work
Supported by the National Key Basic Research and Development of China (Grant Nos. 2006CB943603 and 2006CB503905) and International Collaboration, the Ministry of Science and Technology of China (Grant No. 20070192)

Citation: Li Y, Lin C S, Wang L, et al. Maintenance of human embryonic stem cells on gelatin. Chinese Sci Bull, 2009, 54: 4214—4220, doi: 10.1007/s11434-009-0630-4

in vitro fertilization (IVF). The frozen-thawed embryos were donated by couples after obtaining their informed consent. The protocols used for IVF and hESC derivation were approved by the Ethical Committee of Peking University Health Science Center. The embryos were cultured to the blastocyst stage, and ICMs were isolated by immunosurgery as previously described[15]. Isolated ICMs were cultured on feeder layers of mouse embryonic fibroblasts (MEFs) that were mitotically inactivated by mitomycin C (Sigma). The hES-medium consisted of Knock-out Dulbecco's modified Eagle's medium (Gibco) supplemented with 20% serum replacement (Gibco), 0.1 mmol/L β-mercaptoethanol (Hyclone), 1% nonessential amino acids (Hyclone), 2 mmol/L glutamine (Hyclone), and 4 ng/mL FGF2 (Sigma). hESCs were subcultured every 57 d by enzymatic dissociation using 1 mg/mL collagenase IV (Gibco).

1.2 Preparation of the conditioned hES-medium (CM)

To prepare MEFs for the conditioned hES-medium, MEFs (passage 25) were harvested and were mitotically inactivated by mitomycin C and seeded at 70%—80% confluent density in 60 mm culture dishes in Dulbecco's modi ed Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 2 mmol/L L-glutamine. After at least 10 h, the medium was exchanged with the hES-medium. CM was collected daily and supplemented with an additional 4 ng/mL of bFGF before feeding hESCs. MEFs were again fed with the hES-medium daily and used for 7—10 d for CM collection. The conditioned hES-medium may be used for up to 1 week if stored at 4℃. The CM could also be frozen for storage at –20℃ for two months and thawed for later use with no significant difference from the fresh CM in supporting cell growth.

1.3 Plate coating

Plates were incubated with Matrigel (Becton Dickinson) diluted 1∶20 in cold Knock-out DMEM or gelatin (0.1%) (Sigma) diluted in phosphate-buffered saline (PBS) at room temperature for 1 h.

1.4 Transfer of hESCs from feeder to feeder-free conditions

PUSC-1 was cultured with MEFs for 65 passages, then transferred to Matrigel or gelatin-coated plates. Two different methods were employed for the transfer of the hESCs from feeder cells onto the feeder-free system—on the first is Matrigel—collagenase IV combination and the second a gelatin-trypsin combination. Briefly, the hESC colonies were washed once with PBS, treated with 1 mg/mL collagenase IV for 20—30 min at 37℃ or 0.05% trypsin-EDTA for 1—2 min at 37℃, and gently dissociated to small clumps by pipetting in the culture medium. After washing and resuspension in the conditioned hES-medium, the cells were transferred to the matrigel- or gelatin-coated plates.

1.5 Immunocytochemistry

The following antibodies were used for immunohistochemistry: SSEA-4, TRA-1-60, TRA-1-81, Sox2 (all from Chemicon); and Nanog, SSEA-1, SSEA-3, Oct-4 (all from Santa Cruz Biotechnology). Cells were fixed in 4% paraformaldehyde for 20 min at 4℃, washed with 0.01 mol/L PBS, and incubated with primary antibodies at 4℃ overnight. Mouse or rabbit normal serum was used as a negative control. Localization of antigens was visualized with anti-rabbit or anti-mouse IgG secondary antibodies conjugated with fluorescein (Santa Cruz). In order to detect alkaline phosphatase (AP) activity, the cells were fixed in 4% paraformaldehyde for 20 min at 4℃, rinsed in a buffer of pH 9.5 containing 100 mmol/L Tris-HCl, 100 mmol/L NaCl, and 50 mmol/L $MgCl_2$, and incubated with NBT/BCIP (Roche) in the dark for approximately 20 min. The reaction was stopped after the desired color intensity had been obtained.

1.6 Karyotyping analysis

hESCs (at passage 15 of PUSC-1 and passage 20 of H-1 on gelatin) prepared for karyotype analysis were incubated in a growth medium containing 0.1 mg/mL colcemid for 3—4 h, trypsinized, resuspended in 0.075 mol/L KCl, incubated at 37℃ for 30 min, and then fixed in 3∶1 methanol:acetic acid at RT for 5 min. The centrifugation and fixing steps were repeated three times. Harvested cells were stained using a standard G-banding technique and analyzed using the chromosome pair software (Becton Dickinson).

1.7 Formation of teratomas and staining

hESC colonies cultured on gelatin (at passage 10) were digested with 1 mg/mL collagenase IV into aggregations of approximately 200—400 cells; the cell aggregates were injected into the rear leg muscle of 6 to 8-week- old, severe combined immunodeficient (SCID) mice. Six to eight

weeks later, the resulting tumors were fixed in 4% paraformaldehyde, embedded in paraffin, and examined histologically using hematoxylin and eosin staining.

1.8 Western blot analysis of Oct-4 expression

hESC on matrigel or gelatin (at passage 10) were lysed with lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.5% sodium deoxycholate, 1% NP-40) supplemented with complete protease inhibitors (Roch). Cell lysates (20 mg) were separated by electrophoresis on 10% SDS-polyacrylamide gel and transferred to a NC membrane (Pall). The blot was blocked with TBST (20 mmol/L Tris-HCl, pH 7.6, 136 mmol/L NaCl, and 0.1%Tween-20) containing 5% skim milk and then incubated with the anti-Oct4 antibody (1∶1000, Santa Cruz) or the β-actin antibody (1∶1000, Sigma) at 4℃ overnight. After washing with TBST, the membrane was incubated with the IRDye 700 and 800-conjugated secondary antibody for 1 h at room temperature. The imaging was then performed by the Li-Cor Odyssey Infrared Imaging System.

1.9 Flow cytometry

The hESCs (at passage 12) were trypsinized into single cell suspension and stained with phyco-erythrin (PE) labeled antibody anti-SSEA4 (eBioscience) at 4℃ for 30 min. Mouse IgG2b-PE (Becton Dickinson) was used as the isotype control. Stained cells were analyzed on a FACScaliburTM Flow Cytometer (Becton Dickinson) using CellQuest software.

1.10 Statistical analysis.

All results were expressed as mean±SD. Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls method. A probability of $P<0.05$ was used to indicate a significant difference.

2 Results

2.1 Establishment and characterization of the hESC line: PUSC-1

Six embryos produced by *in vitro* fertilization for clinical purposes were obtained from patients who were fully aware of the scope of this study and signed an informed consent form prior to this study. Inner cell mass (ICM) was isolated from blastcysts by immunosurgery, and one hESC line was derived. These cells were propagated by mechanical dissociation within passage 5 and subsequently by collagenase IV. The resulting hESCs had a high nucleus-to-cytoplasm ratio and prominent nucleoli. The putative ESCs were similar to the hESCs reported previously in morphology and the expression of specific cell markers for pluripotent stem cells (Figure 1(a)－(k)). Our hESC line successfully maintained its undifferentiated morphology after continuous proliferation for more than 70 passages, meanwhile kept a normal female karyotype (Figure 1(l)). The hESCs gave rise to teratomas after being injected into the testes of SCID mice. The resulting teratomas contained tissue representative of all three germ layers (Figure 1(m)－(p)).

2.2 Maintenance of hESCs on gelatin

The hESCs were dissociated by collagenase IV or trypsin treatment and seeded onto matrigel or gelatin. The morphology of the hESCs cultured on gelatin appeared similar to that cultured on matrigel (Figure 2(a) and (b)). The colonies cultured on matrigel or gelatin seemed flatter than those on MEFs (Figure 2(c)). Subsequently, some characterizations of the hESCs cultured on gelatin were performed by examining the expression of undifferentiated markers, karyotype, and differentiation *in vivo*. The hESCs on gelatin were ALP positive (Figure 2(d)) and results of immunoflouresence staining showed that the cells expressed Oct4, Sox2 and SSEA4 (Figure 2(e)－(g)). Their karyotype remained stable (Figure 2(h)) and formed teratomas consisting of differentiated cells and tissue representative from all three germ layers in SCID mice (Figure 2(i)－(l)).

2.3 Comparison between hESCs on matrigel and gelatin

The growth rates of the hESCs cultured in both feeder-free systems were compared by detecting the expression of Oct4, AP and SSEA-4. At passage 10 of the hESCs cultured on matrigel or gelatin, the expression level of Oct4 was detected by Western blot. The data showed that both of these cells expressed a high level of Oct4 (Figure 3(a)).

The hESCs treated by collagenase IV or trypsin were seeded on gelatinor matrigel-coated plates. After 5－6 d, the hESC colonies were stained for AP detection. AP-positive colonies were counted under the microscope field (×200). As shown in Figure 3(b), there is no significant difference in the size and number of AP-

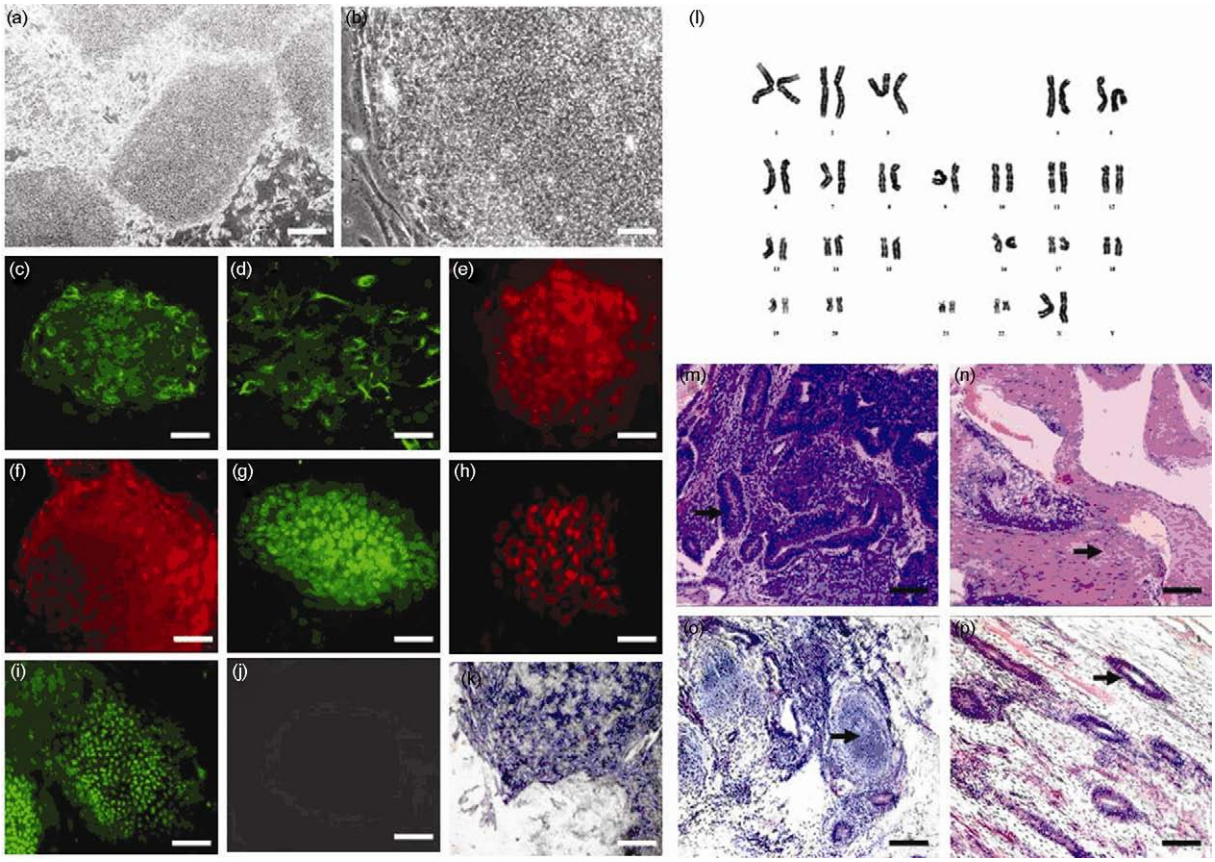

Figure 1 Establishment and characterization of PUSC-1. The flat colonies of hESC are shown in (a). Single ES cells had the characteristics of high nuclear cytoplasm ratio and prominent nucleoli (b). The undifferentiated hESCs were positive for SSEA-3 (c), SSEA-4 (d), TRA-1-60 (e), TRA-1-81 (f), Oct4 (g), Nanog (h) and Sox2 (i); but not by the antibody against SSEA-1(j). The undifferentiated human ES cells also expressed a high level of alkaline phosphotase activity (k). The hESCs maintained normal chromosome complements (l). Teratoma included derivatives from three germ layers including neural epithelium (m), muscle (n), cartilage tissue (o) and glandular epithelium (p). Bar: 150 μm.

positive colonies between the two feeder-free systems (P>0.05) (Figure 3(b)). Moreover, through flow cytometry, no difference was found regarding the ratio of SSEA-4-positive cells cultured on Matrigel and gelatin (95.1% vs. 94.3%, P>0.05) (Figure 3(c)).

2.4 Maintenance of H-1 hESCs on gelatin

The H-1 hESCs were dissociated by trypsin treatment and seeded onto gelatin. The morphology of the H-1 hESCs cultured on gelatin appeared flat (Figure 4(a)). These cells were stained positive for ALP (Figure 4(b)), and results of immunofluoresence staining showed that the cells expressed Oct4, Sox2 and SSEA-4 (Figure 4(c)—(e)). Their karyotype remained stable after growing on gelatin for 20 passages (Figure 4(f)).

3 Discussion

In any attempt to scale up hESCs for the purpose of clinical studies, a defined and cost-effective culture system will be a first irreducible goal[3]. hESCs are anchorage-dependent when growing uniquely as colonies. Current methods to scale them up are therefore focused on the use of matrix[4–6]. In this study we found that, by trypsinization, gelatin is able to support undifferentiated propagation of hESCs for long-term culture. hESCs grown on gelatin expressed a comparable level of Oct, and contained a similar amount of AKP positive colonies and a similar percentage of SSEA-4 positive cells to hESCs grown on matrigel.

Although matrigel is routinely used for feeder-free cultivation of hESCs, its mouse sarcoma origin brought up the safety concerns, the not to mention its costly performance that limits the large-scale production of hESCs. Compared to Matrigel, gelatin is a widely used coating material for cell culture. In spite of being derived from animals, it is regarded as a healthy and safe foodstuff according to the regulation from World Health Organi-

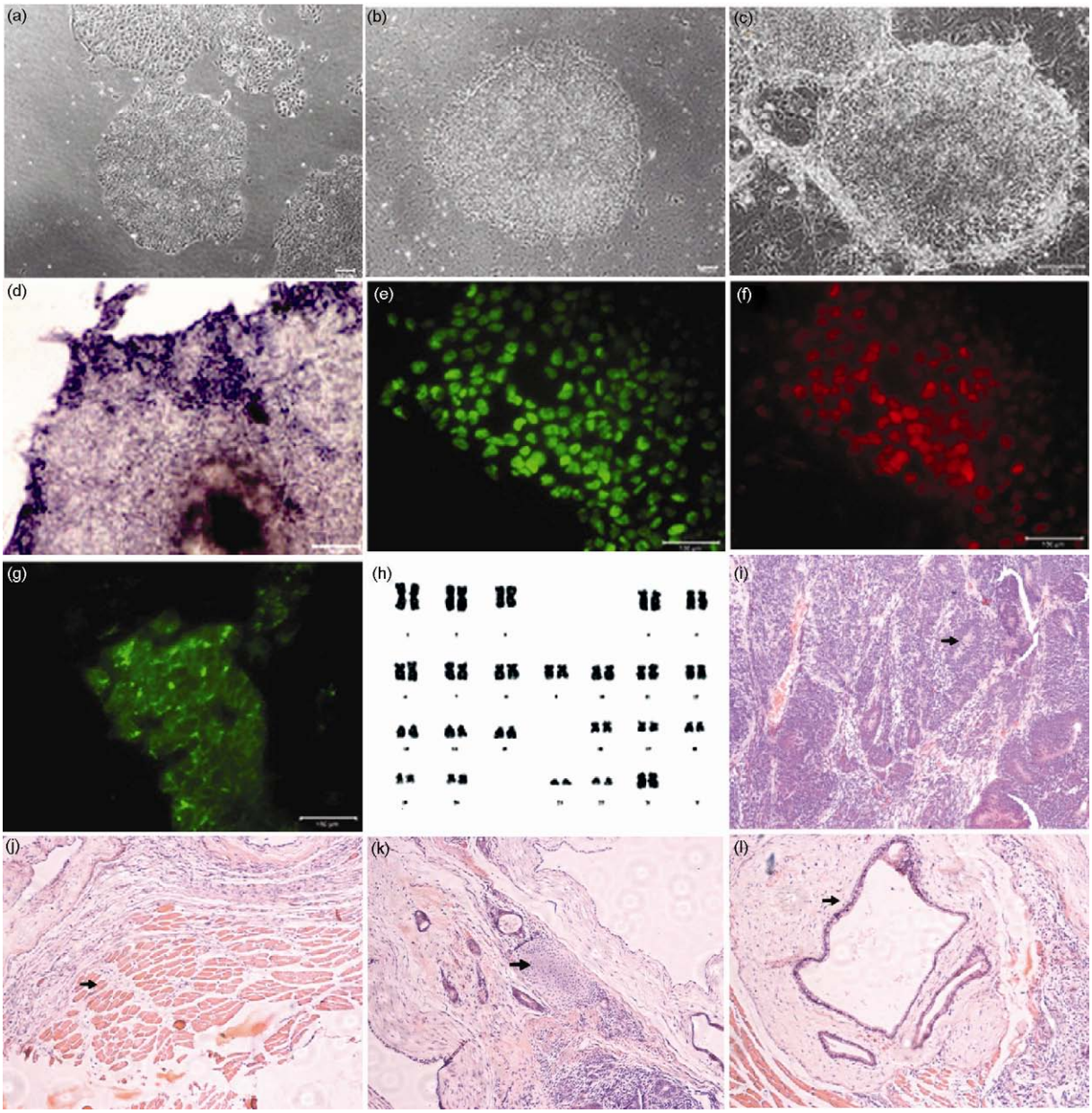

Figure 2 Maintenance of hESCs on gelatin. The morphologies of hESCs cultured on gelatin (a) and on matrigel (b) seemed flatter than those on MEFs (c). The hESCs on gelatin were ALP positive (d); and expressed Oct4 (e), SOX2 (f) and SSEA4 (g). The hESCs still maintained normal chromosome complements on gelatin after 15 passages (h). Teratoma from the hESCs cultured on gelatin included derivatives from three germ layers, including neural epithelium (i), muscle (j), cartilage tissue (k) and glandular epithelium (l). Bar:150 μm.

zation (WHO) and the European Commission for Healthand Consumer Protection (http://www.gelatine.org/en/gelatine/safety/682.htm). Moreover it is almost 10-thousand times cheaper than matrigel.

In this study, we successfully maintained trypsinized hESCs, as well as H1 hESCs on gelatin-coated plate. However, the previous study reported that hES cells dissociated by collagenase VI can not survive on gelatin[11]. Because gelatin is an irreversibly hydrolyzed form of collagen, the possible reason to our success is that gelatin might be a possible substrate of collagenase IV.

It needs further investigation into the possibility that this gelatin-based culturing system is qualified for expansion of hESCs for clinical use. Nonetheless this gelatin-based culture protocol allows us to propagate hESCs in large scale for research purpose.

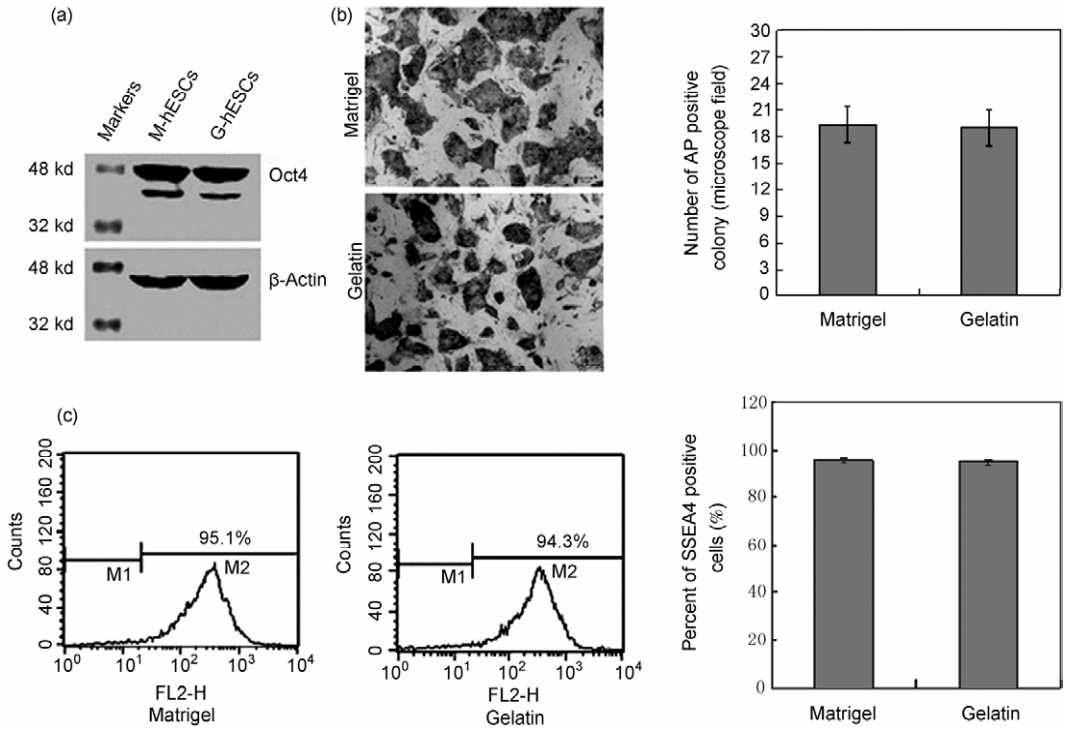

Figure 3 Comparison between hESCs cultured on Matrigel and on gelatin. (a) The expression levels of Oct4 between hESCs on gelatin and hESCs on Matrigel were detected by Western blot; (b) The hESC colonies were stained for AP, and AP-positive colonies were counted under the microscope field (×200). The bar graph shows the mean number of ALP positive clones ± SD for hESCs on matrigel (19.3±1.9) or on gelatin (19±0.8) (*P*=0.65). (c) The percent of SSEA-4-positive cells on gelatin is 94.3%±1.13% as compared with 95.1%±1.24% of that on matrigel (*P*=0.57).

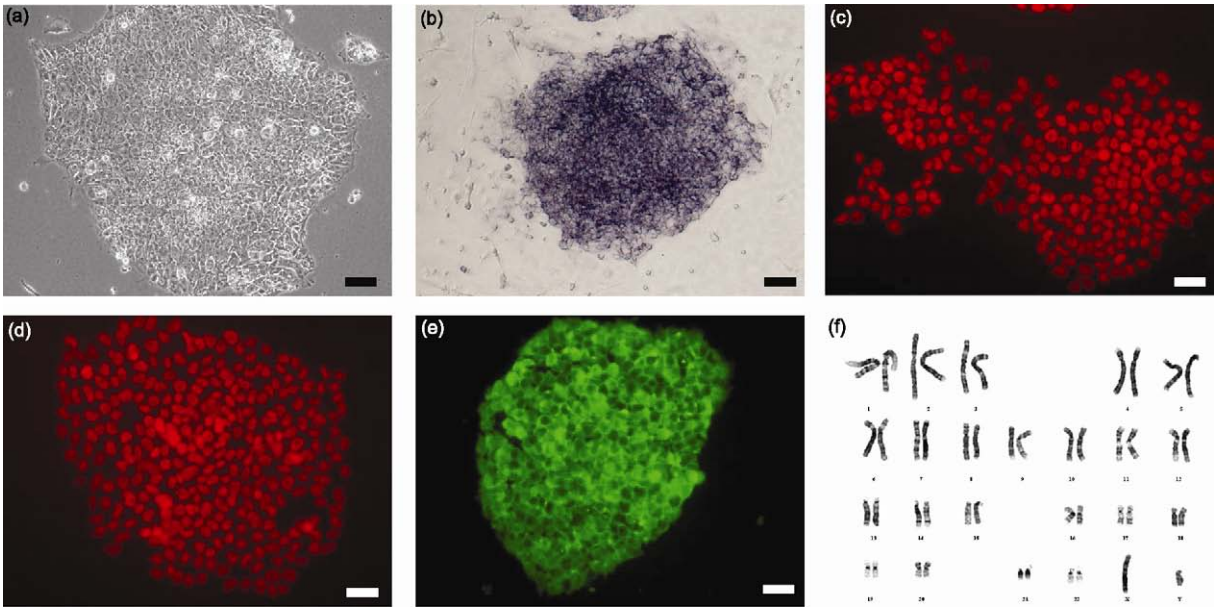

Figure 4 Characterization of H-1 maintained on gelatin. (a) The flat colonies of H-1; (b) the undifferentiated hESCs were positive for alkaline phosphotase; (c) Oct4; (d) Sox2; (e) SSEA-4. The H-1 hESCs maintained normal chromosome complements after growing on gelatin for 20 passages (f). Bar: 150 μm.

1 Reubinoff B E, Pera M F, Fong C Y, et al. Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro*. Nat Biotechnol, 2000, 18: 399－404

2 Thomson J A, Itskovitz-Eldor J, Shapiro S S, et al. Embryonic stem

cell lines derived from human blastocysts. Science, 1998, 282: 1145－1147
3 Bongso A. RMaFCY. From human embryos to clinically compliant embryonic stem cells: Blastocyst culture, xeno-free derivation and cryopreservation, properties and applications of embryonic stem cells. Singapore: World Scientific Publishing Co. Pte. Ltd., 2005
4 Rosler E S, Fisk G J, Ares X, et al. Long-term culture of human embryonic stem cells in feeder-free conditions. Dev Dyn, 2004, 229: 259-274
5 Amit M, Shariki C, Margulets V, et al. Feeder layer- and serum-free culture of human embryonic stem cells. Biol Reprod, 2004, 70: 837－845
6 Sato N, Meijer L, Skaltsounis L, et al. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nat Med, 2004, 10: 55－63
7 Xu R H, Peck R M, Li D S, et al. Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Methods, 2005, 2: 185－190
8 Brimble S N, Zeng X, Weiler D A, et al. Karyotypic stability, genotyping, differentiation, feeder-free maintenance, and gene expression sampling in three human embryonic stem cell lines derived prior to August 9, 2001. Stem Cells Dev, 2004, 13: 585－597
9 Beattie G M, Lopez A D, Bucay N, et al. Activin A maintains pluripotency of human embryonic stem cells in the absence of feeder layers. Stem Cells, 2005, 23: 489－495
10 Klimanskaya I, Chung Y, Meisner L, et al. Human embryonic stem cells derived without feeder cells. Lancet, 2005, 365: 1636－1641
11 Xu C, Inokuma M S, Denham J, et al. Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol, 2001, 19: 971－974
12 Stojkovic P, Lako M, Przyborski S, et al. Human-serum matrix supports undifferentiated growth of human embryonic stem cells. Stem Cells, 2005, 23: 895－902
13 Yim E K, Leong K W. Proliferation and differentiation of human embryonic germ cell derivatives in bioactive polymeric fibrous scaffold. J Biomater Sci Polym Ed, 2005, 16: 1193－1217
14 Li Y J, Chung E H, Rodriguez R T, et al. Hydrogels as artificial matrices for human embryonic stem cell self-renewal. J Biomed Mater Res A, 2006, 79: 1－5
15 Solter D, Knowles B B. Immunosurgery of mouse blastocyst. Proc Natl Acad Sci USA, 1975, 72: 5099－5102