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Comparison of a xeno-free and serum-free culture system for human embryonic stem cells with conventional culture systems

Dan Zhang[†], Qingyun Mai[†], Tao Li, Jia Huang, Chenhui Ding, Mengxi Jia, Canquan Zhou^{*} and Yanwen Xu^{*}

Abstract

Background: Elimination of all animal components during derivation and long-term culture of human embryonic stem cells (hESCs) is necessary for future applications of hESCs in clinical cell therapy.

Methods: In this study, we established the culture system of xeno-free human foreskin fibroblast feeders (XF-HFF) in combination with chemically defined medium (CDM). XF-HFF/CDM was compared with several conventional culture systems. The hESCs cultured in different media were further characterized through karyotype analysis, pluripotency gene expression, and cell differentiation ability.

Results: The hESCs in the XF-HFF/CDM maintained their characteristics including typical morphology and stable karyotype. In addition, hESCs were characterized by fluorescent immunostaining of pluripotent markers and teratoma formation in vivo. RT-PCR analysis shown that the stem cell markers OCT3/4, hTERT, SOX2, and Nanog were present in the cell line hESC-1 grown on XF-HFF/CDM. Furthermore, the results of cell growth and expression of bFGF, Oct-4, and hTERT indicated that XF-HFF/CDM had better performance than human serum-matrix/CDM and XF-HFF/human serum.

Conclusion: The comparison of different xeno-free culture conditions will facilitate clarifying the key features of self-renewal, pluripotency, and derivation and will shed light on clinic applications of hESCs.

Keywords: Human embryonic stem cell, Xeno-free and serum-free culture system, Chemically defined medium, Human foreskin fibroblast cells

Background

Human embryonic stem cells (hESCs) can differentiate into various cell types and possess great potential for cell-replacement therapy including treatment for diabetes, cardiac infarction, and neurodegenerative diseases [1, 2]. The major challenge for the clinical application of hESCs are animal-derived products and undefined factors during in-vitro establishment and expansion of the cells. Nevertheless, hESCs are conventionally cultured on feeder cell layers, of which mouse embryonic fibroblasts (MEFs) are the most common and present several unknown animal-derived products [3]. The risk of graft rejection and

immunoreactions will be increased greatly, demonstrating that a xeno-support system sets a limitation for the therapeutic potential of hESCs to a great extent [4–6]. Optimization and standardization of a fully defined xeno-free culture for hESCs is therefore urgent.

Xeno-free culture conditions continue to evolve to avoid exposure to animal-derived materials [7–9]. However, some issues including chromosomal abnormalities in hESCs arise due to more demanding growth conditions [10]. On the contrary, feeder-free cultures generally present a higher degree of spontaneous differentiation in comparison with conventional cultures, signifying that higher concentration of exogenous basic fibroblast growth factor (bFGF) is required and feeder-free derivation is not optimal for developing transplantable hESC derivatives [11].

Considerable progress has been made towards maintaining hESC cultures since human feeder cells were proposed

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[12]. Several researchers have proved the ability to culture hESCs on human feeder cells which were isolated from human tissues, including adult marrow tissues [13], fetal muscle, fetal skin, human placental fibroblasts [14], and human endometrial cells [15]. Some contributions have been made to derive and culture new human foreskin feeders (hFFs) [16–18].

Other groups have attempted to culture hESCs in chemically defined as well as xeno-free conditions. Some studies have claimed the defined media can retain hESCs successfully in their pluripotent state [19, 20], while other studies have contradicted this [21]. Chin et al. [22] described the defined StemPro and mTeSR1 media without feeder and the xeno-free HEScGRO medium with hFFs for hESC growth. Further efforts should be made to culture hESCs in anticipation of future applications.

In this study, we evaluated whether xeno-free human foreskin fibroblast feeders (XF-HFF) combined with chemically defined medium (CDM) could be successfully used in long-term maintenance of undifferentiated hESCs. At the same time, the characteristics of hESCs grown under these conditions were assessed after prolonged culture, including their unlimited and undifferentiated proliferative ability, maintenance of normal karyotype in long-term culture, and their developmental potential to differentiate into representative tissues of all three embryonic germ layers. In addition, the efficacy of this culture system was compared with other culture conditions with respect to cell growth and undifferentiated states.

Methods

Human material and ethics statement

The design and performance of this study conformed to the ethical standards of the Helsinki Declaration and our national legislation. This study was approved by the Medical Ethical Committee of The First Affiliated Hospital, Sun Yat-sen University. An informed consent form was signed by the participant or the legal guardian for children after receiving a written description of the study.

Human blood was obtained from healthy donors, infant foreskin from circumcised infants, and discarded human embryos from donors undergoing clinical in vitro fertilization treatment. Patients who donated blood and foreskins were negative for HIV, hepatitis B and C, and syphilis.

Preparation of xeno-free human foreskin fibroblast cells (XF-HFF)

Human serum (HS) and XF-HFF were prepared as described previously [1]. XF-HFF were cultured in 90 % Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplied with 10 % HS, 1 %

penicillin–streptomycin (PS; HyClone Laboratories, Inc., Logan, UT, USA). The medium was changed daily. XF-HFF were normally passaged by TrypLE Select (Invitrogen) at a 1:8–1:10 split ratio every 3–5 days.

For preparation of feeder layers, XF-HFF were treated with mitomycin C (Sigma, St. Louis, MO, USA) for 2.5 h, and the treated cells were reseeded at 75,000 cells/cm².

Culture media

Four culture systems were described in this study as follows: XF-HFF combined with CDM (HEScGRO; Chemicon, Billerica, MA, USA) (XF-HFF/CDM group), XF-HFF combined with 20 % HS medium (XF-HFF/HS group), HS-matrix coated on plates without feeder layers combined with CDM (HS-matrix/CDM group), and xeno-contained human foreskin fibroblast feeders (XC-HFF) combined with 20 % KnockOut Serum Replacement (KSR medium) (XC-HFF/KSR group). Two hESC lines (hESC-1, hESC-2) derived in our laboratory were propagated for 25 passages in the XC-HFF/KSR group, and were then transferred to the other three hESC culture systems.

RT-PCR

Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA, USA). Approximately 500 ng of RNA template was reverse-transcribed into cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). The cDNA was amplified by PCR using the Accuprime Taq DNA polymerase system (Invitrogen). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal positive control. PCR products were run on a 2 % agarose gel, stained with ethidium bromide and visualized by UV illumination.

The primers sequences were: octamer-binding transcription factor Oct-4, 5'-GAAGGTATTCAGCCAAAC-3' and 5'-CTTAATCCAAAAACCCTGG-3'; Nanog, 5'-GATCGGGCCCGCCACCATGAGTGTGGATCCAGCTTG-3' and 5'-GATCGAGCTCCATCTTCACACGCTTCAGGTTG-3'; human telomerase reverse transcriptase (hTERT), 5'-CGGAAGAGTGTCTGGAGCAAGT-3' and 5'-GAACAGTGCCTTCACCCTCGA-3'; SOX2, 5'-CGGAAAACCAAGACGCTCA-3' and 5'-GCCGTTTATGTTAGGTTCTGCG-3'; GAPDH, 5'-GTCAGTGGTGACCTGACCT-3' and 5'-CACCACCCTGTTGCTGTAGC-3'.

Karyotype analysis of hESCs

The genetic stability of hESCs grown in the xeno-free culture system was determined by examining the karyotype using a standard G-banding procedure. Briefly, cells were incubated with colcemid, trypsinized, and then resuspended. The samples were then fixed onto the prepared

slides to make the chromosome spreads. The dried slides were baked for 90 min at 80 °C, treated with 0.05 % trypsin for 30 sec to 2 min, and then stained with Giemsa and Leishman's solution.

Teratoma formation

Teratomas were generated by intramuscular injection of undifferentiated hESCs (6×10^6 cells) into the hind limb of severe combined immunodeficient (SCID) mice. The injected mice were sacrificed at 8 weeks post injection, and the resulting teratomas were fixed with 4 % paraformaldehyde and embedded with paraffin. The samples were then stained with hematoxylin and eosin, and observed under a light microscope.

Growth curve analysis

hESCs were plated at a density of 40,000 cells per well and cultured in different medium. The number of cells was counted using a hemacytometer at days 3, 5, and 7 to obtain a growth curve.

Flow cytometry

hESC cultures were rinsed with PBS and then incubated with 0.5 mM EDTA (Sigma) at 37 °C for 5–8 min. The dissociated cells were rinsed twice in PBS for further analysis. Single cells were then blocked with 10 % goat serum (HyClone) for 15 min. All staining procedures were performed using buffer containing 2 % goat serum, 0.1 % sodium azide (Sigma), and 2 mM EDTA in PBS. The blocked cells were incubated with primary antibodies (stage-specific embryonic antigen SSEA-4 or tumor rejection antigen TRA-1-60, 1:20; Chemicon, Temecula, CA) for 30 min at 4 °C followed by three washes in PBS. The cells were then probed with a 1:100 dilution of Alexa-488-conjugated goat F(ab')₂ anti-mouse immunoglobulin (Ig)G₃ or phycoerythrin-conjugated goat F(ab')₂ anti-mouse IgM (both from Invitrogen) for 30 min in the dark at 4 °C. The cells were washed once and resuspended for analysis in staining buffer containing 1 µg/ml of propidium iodide (Sigma) to identify nonviable cells. Flow cytometry analysis was performed using a FACS Calibur flow cytometer (BD Bioscience, San Jose, CA, USA).

Quantification of Oct-4 and hTERT mRNA

RNA was isolated from hESC populations using an RNeasy kit (Qiagen), according to the manufacturer's protocol. Extracted RNA was subsequently treated with DNA-free DNase I (Ambion, Austin, TX, USA). Real-time PCR amplification was performed on an Applied Biosystems International 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The TaqMan one-step RT-PCR master mix (Applied Biosystems) was applied with the following reaction conditions: RT at 48 °C for 30 min; denaturation and AmpliTaq gold activation at

95 °C for 10 min; and amplification for 40 cycles at 95 °C for 15 sec and at 60 °C for 1 min. All reactions were performed in triplicate. The Oct-4 sequences were designed by Applied Biosystems Primer Express software: forward primer, 5'-GCA ACC TGG AGA ATT TGT TCC T-3'; reverse primer, 5'-CCA CAC TCG GAC CAC ATC CT-3'; and probe, FAM-5'-CAG TGC CCG AAA CCC ACA CTG C-3'-TAMRA. The 18 s and hTERT probe and primers were purchased from Applied Biosystems. Relative quantification of gene expression between multiple samples was achieved by normalization against endogenous 18S ribosomal RNA using the $\Delta\Delta CT$ method of quantification (Applied Biosystems). Relative fold difference in gene expression was calculated as $2^{-(\Delta\Delta CT)}$.

Immunofluorescence

The characterization of hESCs grown in animal-free conditions was carried out by measuring the expression of specific pluripotency markers including: SSEA-3, SSEA-4, SSEA-1, TRA-1-60, and TRA-1-81. hESCs were washed once in PBS and fixed in 4 % paraformaldehyde (DGCS biotech) at room temperature for 15 min. The cells were then blocked in PBST (PBS containing 0.1 % Triton 100 (DGCS) and 0.25 % normal donkey serum (Jackson Immuno Research Laboratories, West Baltimore, USA)) at room temperature for 1 h. Primary and secondary antibodies were diluted in PBST. Cells were incubated with primary antibodies at 4 °C for 2 h, followed by three rinses and then incubation with secondary antibodies at room temperature for 1 h. Cells were then washed three times in PBS prior to imaging. Stained preparations were examined under a Nikon Eclipse TE-2000 U fluorescence microscope (Nikon, Tokyo, Japan).

Statistical analysis

All data were obtained from three replicate experiments. Values from all experiments were expressed as the mean \pm standard deviation (SD). Student's *t* test and a chi-square test were employed to analyze the statistical differences between culture groups. The analyses were performed using SPSS software version 10 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

Results

Characterizations of hESCs in the XF-HFF/CDM culture system

hESCs cultured in XF-HFF/CDM exhibit normal cell morphologies

To determine whether hESCs grown in XF-HFF/CDM were maintained in an undifferentiated state, the morphology of hESC-1 cultured in XF-HFF/CDM was examined. As shown in Fig. 1a,b, the hESCs were found to suffer a series of morphological changes after transferring to the

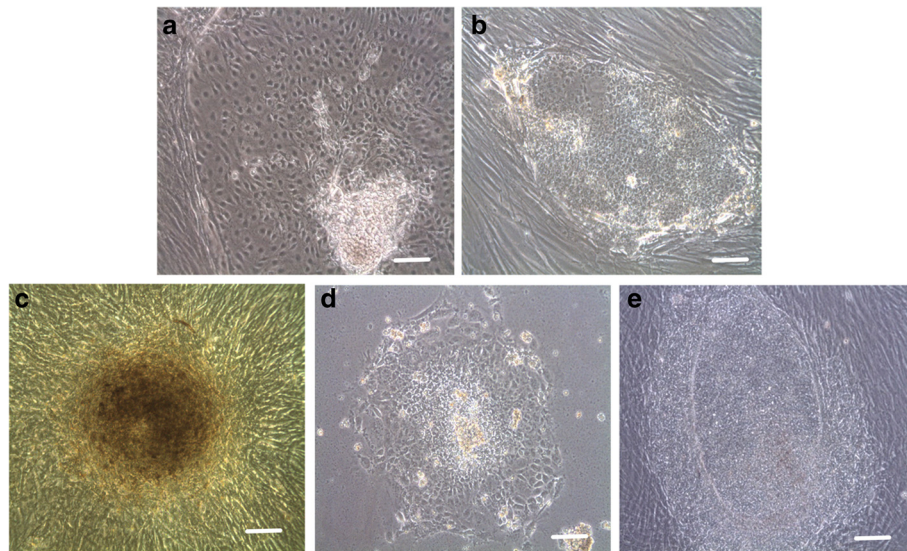


Fig. 1 Morphology of hESC colonies cultured in different conditions. **a** hESC-1 showed a differentiated state after five passages cultured in XF-HFF/CDM conditions. **b** hESC-1 maintained an undifferentiated state cultured in XF-HFF/CDM conditions after 40 passages. **c** Morphology of hESC-1 cultured in XF-HFF/HS medium after five passages is elongated with a high nucleus/cytoplasm ratio, but a low propagation rate. **d** Morphology of hESC-1 cultured on HS-matrix in CDM medium after five passages is elongated with a low nucleus/cytoplasm ratio and propagation rate. **e** Morphology of hESC-1 cells on XC-HFF in KSR medium after 45 passages was still undifferentiated with a high nucleus/cytoplasm ratio and high propagation rate. Scale bar represents 50 μm

new environment. When the undifferentiated colonies were selected and passaged further, the clones gradually adapted to the culture system and regained undifferentiated morphology, tightly packed with sharp edges. The XF-HFF/CDM culture system was able to support maintenance of undifferentiated hESCs for more than 40 passages. The XF-HFF/CDM culture system can thus maintain hESCs in an undifferentiated state in the long term without losing their morphological characteristics.

hESCs cultured in XF-HFF/CDM express stem cell markers

hESCs express stem cell markers that distinguish them from differentiated cells. To confirm that hESCs grown in the XF-HFF/CDM are undifferentiated, this was measured through indirect immunofluorescence staining. The results showed that hESC-1 grown in XF-HFF/CDM expressed the expected stem cell markers SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 (Fig. 2a). Expression of OCT3/4, hTERT, SOX2, and Nanog was further verified by RT-PCR analysis (Fig. 2b). These results indicated that the exogenous factors in XF-HFF/CDM were sufficient for hESC growth in an undifferentiated state.

Karyotype of hESCs cultured in XF-HFF/CDM

Because we demonstrated that hESCs cultured in animal-free XF-HFF/CDM had normal morphological characteristics and expressed stem cell markers, we next determined whether these hESCs retained a normal chromosome complement because hESCs cultured in vitro may lose their

genetic integrity after several passages. To examine the genetic stability of hESCs in XF-HFF/CDM, hESC-1 and hESC-2 cells were karyotyped after >40 passages. The results showed that the hESCs maintained their normal diploid karyotypes (46 XY and 46 XX, respectively) stably after more than 40 consecutive passages (Fig. 3a,b). No major translocations or other chromosomal changes were observed during this period.

hESCs cultured in XF-HFF/CDM are pluripotent

hESCs are pluripotent cells that can differentiate into the three major cell lineages: endodermal, ectodermal, and mesodermal. To further describe the differentiation potential of these hESCs grown in XF-HFF/CDM, we used teratoma formation to observe which tissues would develop from these cultures in vivo. Using histochemical analysis of teratomas after culturing for over 35 passages in the XF-HFF/CDM conditions, we observed the appearance of tissues representing all three germ layers – endoderm (secretory epithelium), ectoderm (skin epithelium), and mesoderm (cartilage) (Fig. 4). These tissues contained multiple cell types from each of the major cell lineages. Hence, the cells cultured in XF-HFF/CDM maintained their pluripotency in vivo.

Comparison of different hESC culture systems

Recently, a xeno-free and feeder-free culture system based on HS-matrix and CDM or a cofeeder culture system based on xeno-free feeder cells and HS medium has

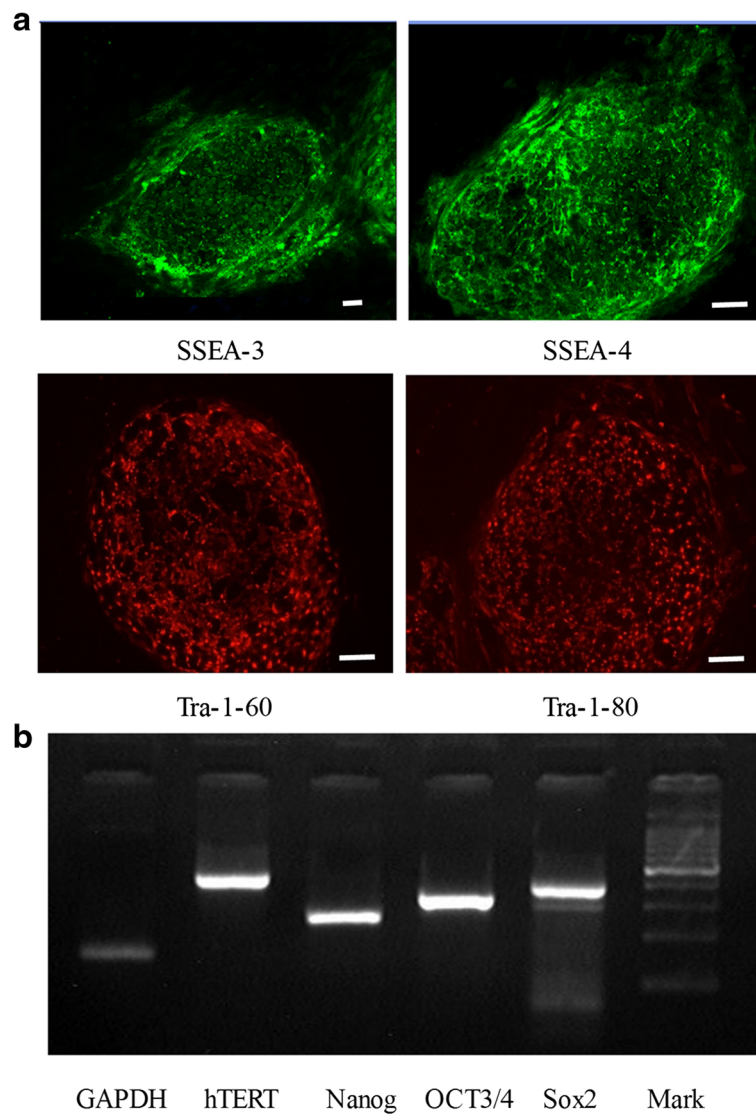
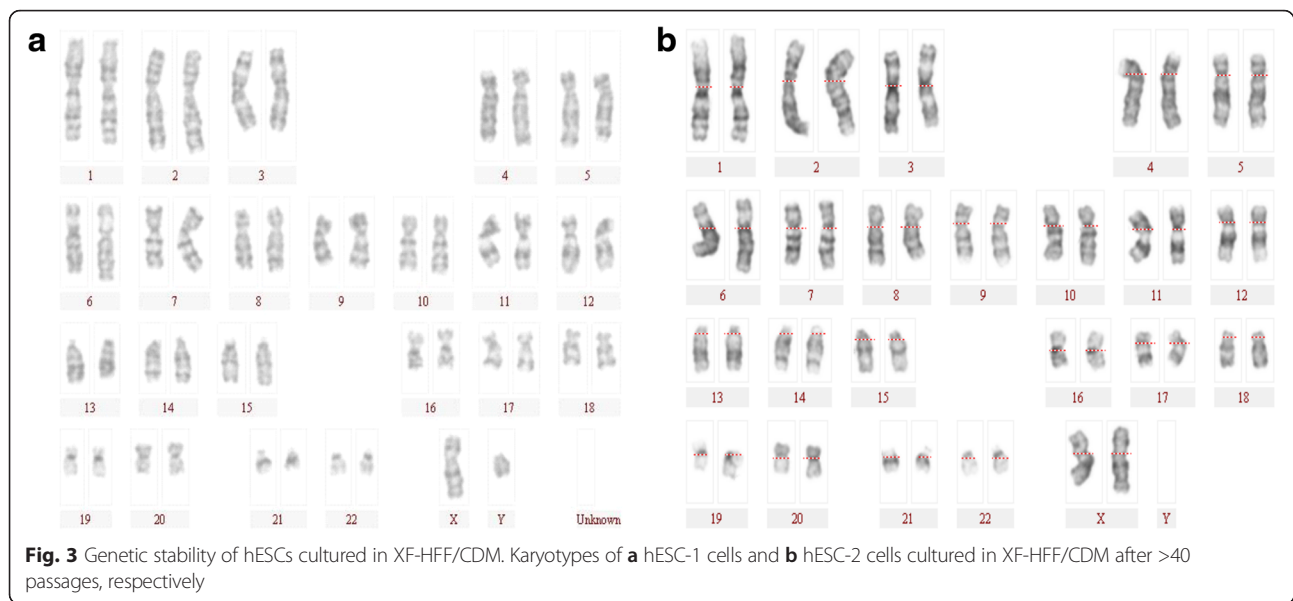


Fig. 2 Expression of stem cell markers in hESC-1 grown on XF-HFF in CDM conditions after >30 passages. **a** Immunofluorescence staining with antibodies of stem cell markers: SSEA3 (upper left), SSEA4 (upper left), TRA-1-60 (lower left), and TRA-1-81 (lower right). **b** RT-PCR analysis of the expression of stem cell markers OCT3/4, hTERT, SOX2, and Nanog, with GAPDH as positive control. Scale bar represents 50 μ m. GAPDH glyceraldehyde-3-phosphate dehydrogenase, hTERT human telomerase reverse transcriptase, OCT octamer-binding transcription factor, SSEA stage-specific embryonic antigen, Tra tumor rejection antigen

been developed. To further assess their culture efficacy, the characteristics of hESCs grown in XF-HFF/CDM were compared with XF-HFF/HS and HS-matrix/CDM. Two hESC lines were transferred from the original XC-HFF/KSR culture conditions to other three culture systems. As shown in Table 1, after transferring four passages the hESCs in XF-HFF/CDM adapted to the new environment quickly, and their survival rate was similar to that in XF-HFF/KSR. Furthermore, the differentiation rate was far lower than that of HS-matrix/CDM and XF-HFF/HS. These results demonstrate that it is easier to meet the requirements for hESC growth in the XF-HFF/CDM system.

Morphology of hESCs cultured with the other three culture media

Based on morphology, the colonies in XF-HFF/HS and HS-matrix/CDM became thinner and some lost their regular shapes and defined borders compared with that seen in XC-HFF/KSR by bright-field microscopy (Fig. 1c–e). In the XF-HFF/HS group and the HS-matrix/CDM group, hESCs lost their undifferentiated morphology and showed a high rate of differentiation and a low rate of colony formation (Fig. 1c,d). In addition, they were unable to maintain their undifferentiated states over six passages. The results indicated that XF-HFF/CDM had an advantage over XF-HFF/HS



and HS-matrix/CDM in maintaining the pluripotency of hESCs.

Growth of hESCs cultured with the other three culture media

To further understand the growth rate of hESCs, the cell number was calculated at days 3, 5, and 7 after transferring from the initial XC-HFF/KSR condition. As shown in Fig. 5a, the rate of cell proliferation in XF-HFF/CDM was obviously higher than that in XF-HFF/HS and HS-matrix/CDM, which had a similar trend to the growth curve in XC-HFF/KSR. The analysis verified that XF-HFF/CDM was superior to HS-matrix/CDM and XF-HFF/HS systems in supporting the growth of hESCs.

Undifferentiated states of hESC clones under different culture systems

The undifferentiated states of hESC clones under different culture systems were compared by analyzing the expression

levels of SSEA-4 and TRA-1-60 by flow cytometry (Fig. 5b). Consistent with the morphologic observations, both CDM-cultured and KSR medium-cultured hESC clones on hFFs expressed high levels of undifferentiated surface marker expression. hESCs cultured in HS-matrix with CDM or HS medium on hFFs, however, downregulated these markers.

We also examined the concentrations of bFGF in each hESC culture medium. After incubation at 37 °C for 24 h, we observed a significant reduction in bFGF levels in all cultures ($p < 0.05$), but the reduction was greater in medium from feeder-free conditions compared with that in medium from cultures with feeder cells (18.2 % vs 36.8–63.8 %) (Fig. 6a).

We performed quantitative RT (qRT)-PCR to confirm the expression levels of hESC markers. OCT-4 and hTERT were expressed in undifferentiated hESCs and downregulated upon differentiation. Figure 6b shows that hESCs in HS-matrix conditions or HS medium

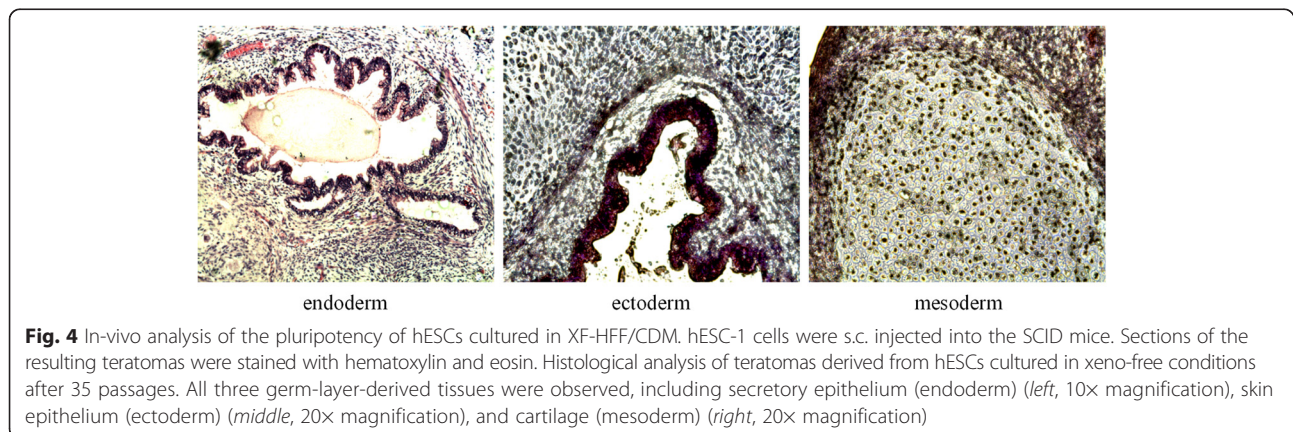


Table 1 Culture efficacy of XF-HFF/CDM conditions and other culture conditions on the survival and differentiation rates of hESCs after transferring four passages

Group	Colony formation rate (%) ^a	Differentiation rate (%) ^b
XF-HFF/CDM(P ₂₅₊₄₀)	91.25 ± 2.22	6.00 ± 1.83
HS-matrix/CDM(P ₂₅₊₄₀)	26.5 ± 2.08*	85.00 ± 3.60*
XF-HFF/HS(P ₂₅₊₄₀)	77.75 ± 2.21*	86.00 ± 3.37*
XF-HFF/KSR(P ₂₅₊₄₀)	93.75 ± 2.22	5.50 ± 2.02

^aNumber of hESC colonies/total number of hESC colonies

^bNumber of hESC colonies with partial or whole differentiation/total number of hESC colonies

**p* < 0.01, control groups versus new culture condition group

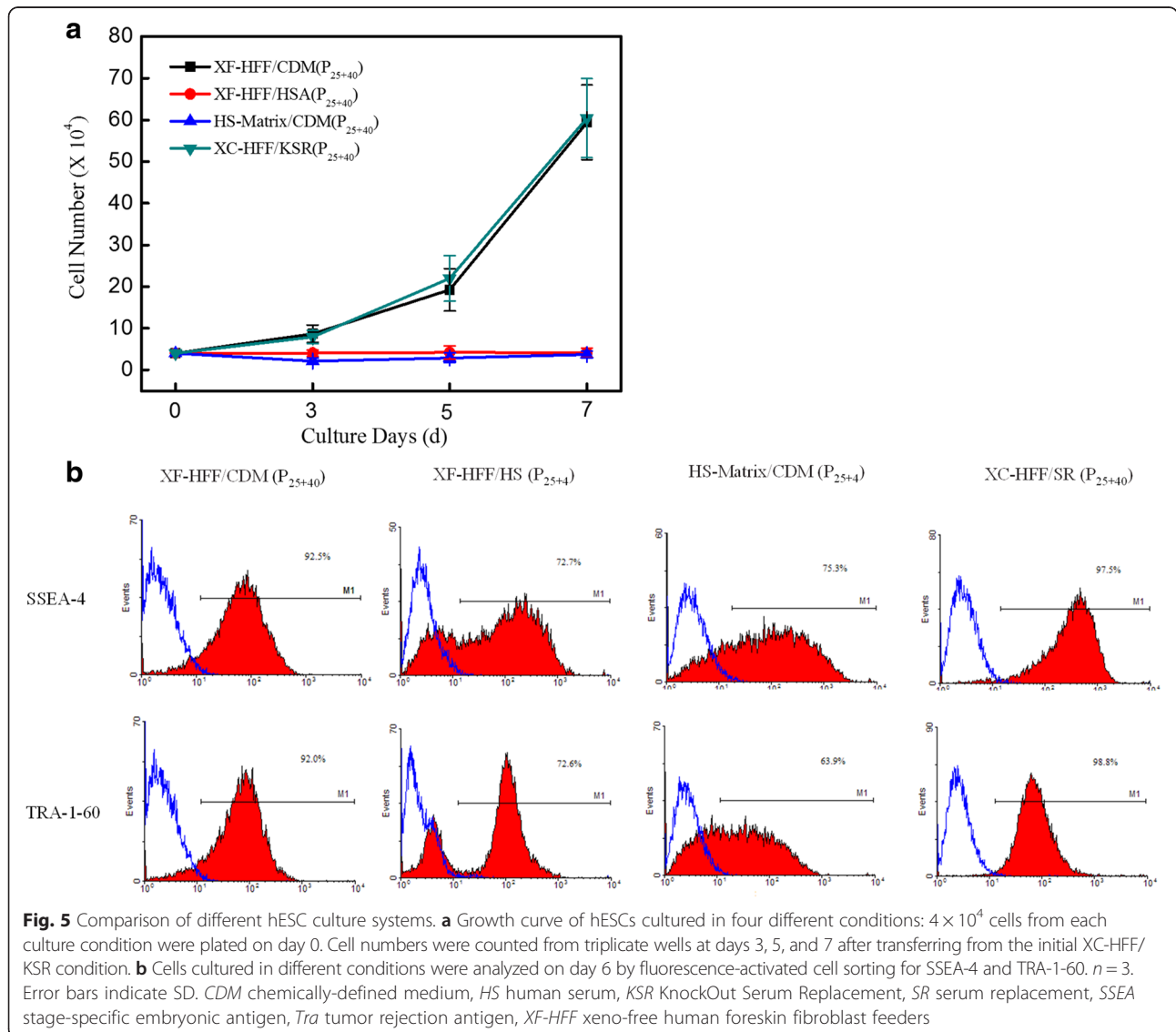
CDM chemically-defined medium, hESC human embryonic stem cell, HS human serum, KSR KnockOut Serum Replacement, XF-HFF xeno-free human foreskin fibroblast feeders

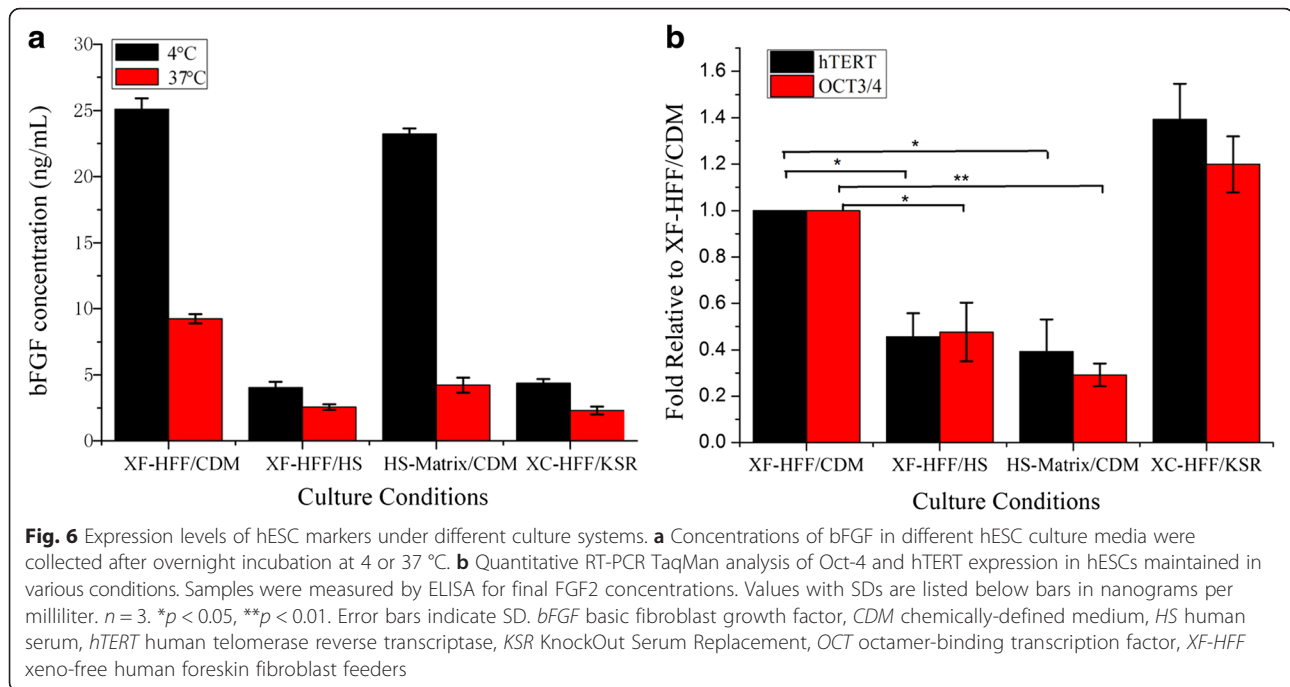
P25+40, defined as the embryonic stem cells which were established and cultured in XC-HFF/KSR culture system for 25 passages, then were shifted to the culture systems of experimental group and control group for 40 passages

conditions expressed low levels of OCT-4 and hTERT. The expression level of the pluripotency marker OCT-4 under XF-HFF/CDM was twofold higher than that under HS-matrix conditions or HS medium conditions. Measurement of hTERT expression by RT-PCR in four culture systems showed that the changing trend was almost the same as that of OCT-4 (Fig. 6b). The hESCs maintained in the XF-HFF/CDM system therefore had relatively high levels of OCT-4 and hTERT, resembling undifferentiated hESCs maintained on XC-HFF/KSR.

Discussion

As a renewable cell source for future regenerative medicine applications, the derivation, passaging, and culture of hESCs have attracted widespread attention [23]. To generate therapeutically safe and usable hESC derivatives for clinical cell treatment, all animal-derived materials





which are a potential risk for infection transmitted by animal pathogens must be eliminated during the process [24].

The major sources of xeno-contamination are the presence of animal feeders and the use of either FBS or serum replacement (SR) in the culture medium [4]. Many groups have previously claimed successful culture of hESCs on hFFs and other feeders [7, 12, 14, 25–28]. Hovatta et al. [27] used commercially available human foreskin fibroblasts for hESC propagation. Crook et al. [29] proposed the proof of concept for the generation of six clinical-grade hESC lines using hFFs as feeder cells. hFFs isolated from infant foreskin have a distinct advantage over MEFs. In addition to the xenobiotic issue, hFFs can be cultured for up to 62 passages before senescence, while MFFs can only be propagated for five passages [7, 30]. Moreover, little ethical concern is considered over the acquirement of foreskin tissue for the generation of feeder cells.

Another obstacle to overcome was the presence of FBS or SR in the culture medium. Several studies described the use of HS as serum supplement to culture hESCs. However, it has been proved previously that commercial xeno-free SRs and human sera are inappropriate for long-term culture of hESCs [21]. Some attempts to use HS as a medium supplement failed due to spontaneous differentiation [12, 26, 31]. It is important to point out that HS may be involved in the interactions between secreted factors from the feeder cells. Therefore, it is highly desirable to employ defined components rather than complex mixtures [32–35]. The establishment of a chemically defined xeno-free and feeder-free

culture system is advantageous for exact identification of factors secreted by feeder layers or signal pathways existing in them. Thus, xeno-free commercially available CDM was employed in the culture system.

Our data demonstrate that hESCs can be successfully derived and cultured long term in the XF-HFF/CDM system while maintaining their undifferentiated state. Furthermore, teratoma analysis demonstrated that hESCs maintain their pluripotency and differentiation potential, which were comparable with hESCs cultured in the conventional hESC culture medium. The use of CDM with hFF cells has been described previously [17]. On the basis of that work, we further presented the differences of culture systems for hESCs. We tested four culture systems and two hESC lines, and little variation was observed between hESCs cultured in CDM and KSR medium in maintaining undifferentiated states of hESCs. On the other hand, data obtained by flow cytometry and qRT-PCR demonstrated that feeder cells supported the growth of undifferentiated hESCs more efficiently than HS-matrix when cells were presented in CDM. The HS-matrix, which contains hyaluronic acid, fibronectin, and vitronectin, as well as other unknown human factors, supports the attachment and growth of undifferentiated cells [36]. Stojkovic et al. [37] suggested that HS used as a matrix maintained pluripotency and genomic stability of hESCs. Meng et al. [38] pointed that extracellular matrix isolated from foreskin fibroblasts has an advantage in the process of long-term xeno-free hESC culture. However, feeder-free cultures usually presented a higher degree of spontaneous differentiation than conventional

culture along with a higher concentration of exogenous bFGF, which indicated that feeder-free derivation and culture were not suitable for developing transplantable hESC derivatives [11]. The results in this manuscript also proved that the HS-matrix/CDM system could not maintain the undifferentiated state and differentiated potential of hESCs. Although the molecular and developmental mechanisms controlling the pluripotency and differentiation of hESCs are largely unknown, feeder cells may provide a better, more stable environment, and thus they can secrete the unique proteins that participate in cell growth, extracellular matrix formation, and remodeling [1, 39].

The results demonstrated that CDM provided an effective replacement for HS medium, comparable with KSR medium, to support hESC self-renewal and pluripotency. This observation suggests that CDM and KSR medium culture conditions supply similar growth factors or signal pathways for hESC growth. CDM is more suitable than serum-containing medium for studies that aimed at identifying the exogenous signals required for undifferentiated hESC growth. XF-HFF/HS were unable to maintain undifferentiated hESCs in long-term growth.

Exogenous bFGF has been identified as a key factor involved in self-renewal of hESCs [40–43], and some studies have indicated that FGF proteins are sensitive to thermal denaturation, while fibroblasts likely secrete either protease inhibitors or binding proteins that modulate bFGF stability [42, 44]. In our work, we found that bFGF was unstable in 37 °C culture conditions. Although the concentrations of bFGF declined in all culture conditions, they were higher in co-feeder conditions than in feeder-free conditions. The data indicated that fibroblast is likely to secrete some proteins which modulate the stability of bFGF [42], and also indicated that the stability of bFGF was not the only factor responsible for the successful culture of hESCs.

Lysophosphatidic acid (LPA) as the key lipid is a simple phospholipid mediator which plays an important role in the regulation of cell proliferation, migration, and survival of multiple cell types [45, 46]. Todorova et al. [47] proposed that LPA was involved in the expression of the Ca²⁺-dependent early response gene *c-myc*, which was a key gene implicated in ESC self-renewal and pluripotency. Therefore, LPA increased the proliferation rate of mESCs. Liu et al. demonstrated that LPA induced the expression of the erythroid biomarkers in cultured human hematopoietic stem cells (hHSCs) under plasma-free conditions. In addition, LPA was also reported to enhance osteogenic differentiation of human mesenchymal stem cells [48]. These results suggested that LPA may play a critical role in cell fate determination. Hence, we suspected that HFF-1 cells could secrete LPA to support the long-term culture.

Conclusion

We have reported the development of a xeno-free and serum-free method for culturing hESCs. The hESCs in the established XF-HFF/CDM culture system maintained their properties, such as typical morphology, pluripotency, capacity to form three germ layers *in vivo*, and stable karyotype. The results show that the XF-HFF/CDM culture medium is applicable for further optimization of xeno-free establishment, culture, and differentiation of stem cells. Future studies should focus on the comparison of different xeno-free culture conditions and on validation of these conditions to demonstrate the key features of self-renewal, pluripotency, and derivation.

Abbreviations

bFGF, basic fibroblast growth factor; CDM, chemically defined medium; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent-based assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hESC, human embryonic stem cell; hFF, human foreskin feeder; HS, human serum; hTERT, human telomerase reverse transcriptase; KSR, KnockOut Serum Replacement; LPA, lysophosphatidic acid; MEF, mouse embryonic fibroblast; OCT, octamer-binding transcription factor; qRT-PCR, quantitative RT-PCR; SCID, severe combined immunodeficient; SR, serum replacement; SSEA, stage-specific embryonic antigen; Tra, tumor rejection antigen; XC-HFF, xeno-contained human foreskin fibroblast feeders; XF-HFF, xeno-free human foreskin fibroblast feeders

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Authors' contributions

DZ carried out the teratoma formation studies, participated in the quantification of Oct-4 and hTERT mRNA assays, and drafted the manuscript. QM carried out the immunofluorescence assays and revised the manuscript. TL participated in the quantification of Oct-4 and hTERT mRNA assays and drafted the manuscript. JH carried out the karyotype of hESC studies and drafted the manuscript. DH participated in growth curve analysis and revised the manuscript. BC and participated in the design of the study, performed the statistical analysis, and helped to revise the manuscript. CZ and YX conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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