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ORIGINAL PAPER

Development of a simple, repeatable, and cost-effective extracellular matrix for long-term xeno-free and feeder-free self-renewal of human pluripotent stem cells

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Abstract Given the potential importance of human pluripotent stem cells (hPSCs) in translational research and regenerative medicine, the aim of the present study was to develop a simple, safe, and cost-effective substrate to expand hPSCs. We report the development of an extracellular matrix (ECM), designated "RoGel," based on conditioned medium (CM) of human fibroblasts under serum- and xeno-free culture conditions. The long-term self-renewal of hPSCs on RoGel was also assessed. The results showed that self-renewal, pluripotency, plating efficiency, and cloning efficiency of hPSCs on this newly developed ECM were similar to those of Matrigel, the conventional mouse-cell line-derived ECM. The cells had the capability to passage mechanically on a cold surface, which resulted in their long-term maintenance with normal karyotype. We have demonstrated that CM-coated plates preserved for 1 year at room temperature maintained the capability of hPSC expansion. This ECM provides an attractive hPSC culture platform for both research and future therapeutic applications.

Keywords Conditioned medium · Extracellular matrix · Human-induced pluripotent stem cells · Human embryonic stem cells · Self-renewal

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Introduction

Since the first report on the generation of human embryonic stem cells (hESCs) from preimplantation embryos (Thomson et al. 1998) and human-induced pluripotent stem cells (hiPSCs) from reprogramming somatic cells (Yu et al. 2007; Takahashi et al. 2007), there has been tremendous interest in developing culture systems that decrease cell heterogeneity and produce cells at a quality and scale suitable for biomedical applications. These human pluripotent stem cells (hPSCs) have provided fascinating possibilities and tools for the study of human development and genetic diseases, in addition to developing toxicological and pharmaceutical applications, as well as in vitro disease modeling [for review see (Klimanskaya et al. 2008)]. Until now, various culture conditions from mouse or human feeder cell layers to feeder- and serum-free conditions have been developed for self-renewal and maintenance of pluripotency (Rajala et al. 2007).

Progress in developing culture media for hESC expansion has led to the production of several media, such as X-VIVO 10 (Genbacev et al. 2005), mTeSR (Ludwig et al. 2006), and STEMPRO (Heng et al. 2012). In contrast, it remains a challenge to identify a simple, repeatable, cost-effective, and optimum substratum for the propagation of hPSCs in feeder-free conditions. The typical extracellular matrix (ECM) used is Matrigel (MG; Totonchi et al. 2010; Genbacev et al. 2005; Carpenter et al. 2004; Wang et al. 2005; Xu et al. 2005), a complex mouse sarcoma cell basement membrane extract that consists of various ECM

proteins and growth factors (Hughes et al. 2010). MG is the sole gold standard ECM for long-term feeder-free expansion of undifferentiated hPSCs. As alternatives to MG, several research groups have used laminin (Xu et al. 2001; Beattie et al. 2005); fibronectin (Amit et al. 2004); vitronectin (Braam et al. 2008); animal-derived matrices (Brafman et al. 2009); ECM derived from mouse embryonic fibroblasts (Klimanskaya et al. 2005) and human foreskin fibroblast (HFF; Meng et al. 2010); a mixture of human collagen IV, vitronectin, fibronectin, and laminin (Rajala et al. 2007); and human serum coating (Stojkovic et al. 2005) as replacements for feeder cells in hESC cultures. Feeder-free derivation of hESC lines has been successfully described using a mouse-derived matrix (Klimanskaya et al. 2005) or a combination of human laminin, collagen IV, fibronectin, and vitronectin matrix (Ludwig et al. 2006).

However, most of these biological materials have limited scalability, lack availability in laboratories, may have high batch-to-batch variability, or are not cost-effective for routine culture [for review see (Villa-Diaz et al. 2013)]. In addition, animal-derived materials expose hPSCs to potentially hazardous pathogens, which allows for the transfer of immunogenic epitopes (Martin and Vermette 2005). Most of these ECMs are generally effective for short-term propagation. Researchers are still searching for xeno-free culture vessel coatings that do not induce cellular differentiation in hPSCs and save chromosomal integrity (Draper et al. 2004; Ludwig et al. 2006; Mitalipova et al. 2005).

Recently, it was reported that hypotonic lysis of human foreskin fibroblasts produces a human ECM for hESC expansion (Escobedo-Lucea et al. 2012). Additionally, several groups have reported that feeder-free propagation of hESCs is achievable with recombinant human laminin 511 (Rodin et al. 2010; Domogatskaya et al. 2008), recombinant E8 fragments of laminin isoforms (LM-E8s; Miyazaki et al. 2012), a defined glycosaminoglycan-binding substratum (GKKQRFRHRNRKG; Klim et al. 2010), and peptides derived from the RGD-containing adhesion domains of bone sialoprotein and vitronectin, which are covalently linked to an acrylate surface (Melkoumian et al. 2010). The monomers with high acrylate content have a moderate wettability and employ integrin α5β3 and α5β5 engagement with adsorbed vitronectin (Mei et al. 2010), synthetic polymer substrates, named poly[2-(methacryloyloxy) ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide] (PMEDSAH; Villa-Diaz et al. 2010) and poly(methyl vinyl ether-alt-maleic anhydride; Brafman et al. 2010), hydrogel interfaces of aminopropylmethacrylamide (APMAAm; Irwin et al. 2011), UV/ozone radiation modification of typical cell culture plastics (Saha et al. 2011), and a chemically defined thermoresponsive synthetic hydrogel based on 2-(diethylamino) ethyl acrylate (Zhang et al. 2013). Nevertheless, these materials have most of the aforementioned problems for long-term routine expansion of hPSCs in addition to technical difficulties in the synthesis of their components. Therefore, attempts to find substrates suitable for simple promotion of long-term hESC and hiPSC self-renewal have attracted great interest.

Here, we describe the development of a safe, economical ECM, designated "RoGel," that is made of conditioned medium (CM) of human fibroblasts under serum- and xeno-free culture conditions. We subsequently evaluated the long-term self-renewal of hESCs and hiPSCs in this ECM by using a variety of experimental approaches.

Materials and methods

Culturing hESCs and hiPSCs

We used hESC lines (Royan H5 and Royan H6; Baharvand et al. 2006) and hiPSC lines (hiPSC1 and hiPSC4; Totonchi et al. 2010) in this study. Prior to this study, the cell lines were passaged and maintained on MG (0.3 mg/ml; Sigma-Aldrich, E1270) under feeder-free culture conditions in an hESC medium that contained DMEM/F12 medium (Invitrogen, 21331-020) supplemented with 20 % KnockOut™ serum replacement XenoFree CTS™ (KOSR; Invitrogen, 12618-013), 2 mM L-glutamine (L-Gln; Invitrogen, 25030-081), 0.1 mM β-mercaptoethanol (β-ME; Sigma-Aldrich, M7522), 1 % nonessential amino acids (NEAAs; Invitrogen, 11140-035), 1 % penicillin and streptomycin (Invitrogen, 15070-063), 1 % insulin-transferrin-selenite (ITS; Invitrogen, 41400-045), and 100 ng/ml basic fibroblast growth factor (bFGF; Royan Institute). The cells were grown in 5 % CO_2 at 95 % humidity.

Experimental design

The experimental design and assessments for the development of an ECM for the long-term culture of undifferentiated hESCs and hiPSCs are described in Fig. 1. MG was used as a positive control in all experiments. Initially (prephase step), we examined whether DMEM/F12 or neurobasal medium could support the plating efficiency (PE) of hPSCs. Therefore, MG was diluted in DMEM/F12 as a conventional medium for hESC culture or in neurobasal medium (Invitrogen, 21103049).

Next, in the first phase we prepared the CMs with a medium on low-density human dermal fibroblast (HDF) for a 24-h culture. The medium was supplemented with L-Gln, β-ME, NEAAs, and ITS. The feeder cell layers were inactivated by mitomycin C (10 μg/ml, Sigma-Aldrich, M0503) for 2 h. The CMs were prepared by either 24-h or 72-h incubation of medium on HDFs. The feeder cells were cultured at high (50,000 cells/cm^2) or low (5,000

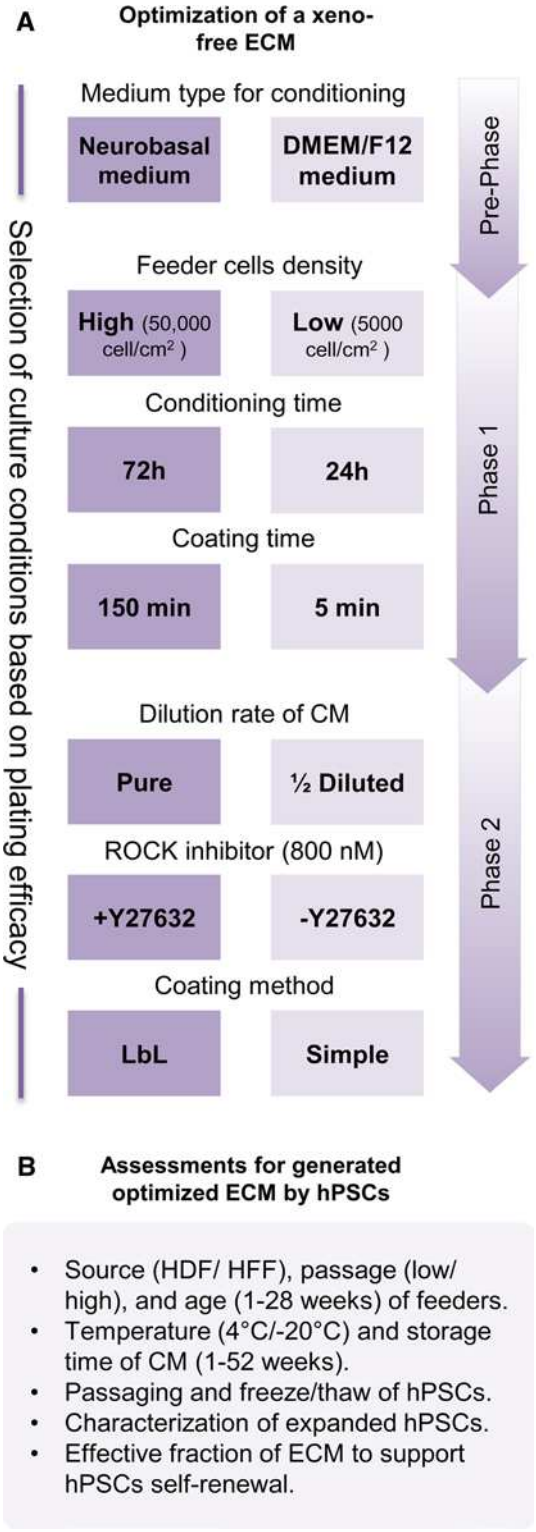

Fig. 1 The experimental design and assessments for the development of an ECM for long-term culture of undifferentiated hPSCs. **a** Optimization of the CM of human fibroblasts as an ECM. **b** Evaluations for produced optimized ECM by hPSCs. See "Materials and methods" for more details

cells/cm^2) densities in 25 cm^2 T flasks (TPP, 90025) in 4 ml of medium (160 μl/cm^2). To coat, plates were exposed to CMs for either 5 min or 150 min. These conditions were compared using PE [= (the number of ALP-positive colonies/number of seeded explants) × 100] on the seventh day after passage.

After the selection of a comparable group with MG in the first phase, for the second phase we culture hPSCs in pure or 50 % diluted CM in the presence or absence of the ROCK inhibitor, Y-27632 dihydrochloride monohydrate (ROCKi, 1 μM; Sigma-Aldrich, Y0503). The plates were then coated using a simple or layer-by-layer coating method.

To coat each well of a six-well plate for "simple coating," 1 ml CM was added and swirled to spread the CM across the surface. The plates were then incubated at 37 °C. After 150 min, the extra CM was removed and plates were allowed to dry at room temperature for about 15–20 min. To achieve layer-by-layer coating, we recoated the plates using the same procedure after the first coating was allowed to dry. Prior to cell seeding, plates were rinsed with Dulbecco's phosphate-buffered saline (DPBS; Invitrogen, 14040-117) that contained Ca^{2+} and Mg^{2+}.

Passaging of human pluripotent stem cells

For passaging, the cells were washed once with DPBS and then incubated with DMEM/F12 that consisted of 1:1 collagenase IV (1 mg/ml; Invitrogen, 17104-019) and dispase (2 mg/ml; Invitrogen, 17105-041) at 37 °C for 3–5 min. When the colonies at the edge of the plates began to dissociate from the bottom, the enzyme was removed and plates were washed twice with DPBS. Cells were collected by gentle pipetting and then replated on MG (Sigma-Aldrich, E1270)- or CM-coated vessels. The medium was changed every other day.

Immunofluorescence and alkaline phosphatase (ALK) staining

The cells were fixed in 4 % paraformaldehyde for 20 min, permeabilized with 0.2 % triton X-100 for 30 min, and blocked in 10 % goat serum in PBS for 1 h. Cells were incubated with the appropriate primary antibody for 1 h at 37 °C, washed with PBS, and incubated with FITC-conjugated secondary antibodies anti-mouse IgM (1:100, Sigma-Aldrich, F9259), anti-rat IgM (1:200, eBioscience, 11-0990), and anti-mouse IgG (1:200, Sigma-Aldrich, F9006) as appropriate for 30 min at 37 °C.

The primary antibodies were anti-TRA-1-81 (1:100, Chemicon, MAB4381), OCT4 (1:100, Santa Cruz Biotechnology, SC-5279), and SSEA-3 (1:50, Chemicon,

MAB4303) for undifferentiated hESC and hiPSC determination. Mouse anti-TUJ1 (1:200, Sigma-Aldrich, T8660) was used to stain mature neuron cells from hESCs. Nuclei were counterstained with DAPI (0.1 μg/ml, Sigma-Aldrich, D8417) or propidium iodide (Sigma-Aldrich, P4864). The cells were analyzed with a fluorescent microscope (Olympus, Japan).

ALK staining was performed based on the manufacturer's recommendations (Sigma-Aldrich, 86R).

Flow cytometric analysis

The Ki-67 antigen, an excellent marker to exhibit cell proliferation of hPSCs cultured on different substrates, was analyzed by flow cytometry using the monoclonal mouse anti-human Ki-67 antigen, clone Ki-67 (F0788, Dako Cytomation). For the purpose of quantization of the pluripotent status of hESCs after culture under various conditions (two different substrates combined with two different media), we analyzed cells by flow cytometry for NANOG (1:500, Sigma-Aldrich, N3038). In order to show the pluripotency status of hPSCs cultured on dissimilar substrates and at various passage numbers, we examined these cells by flow cytometry for NANOG and OCT4. The matched secondary antibody was Alexa Fluor® 488 goat anti-mouse IgG (1:700, Invitrogen, A-11001). Briefly, hPSCs cultured on 6-cm dishes (BD Falcon™ 60 mm Easy-Grip™ Cell Culture Dish, BD, 353004) were coated with MG or CM derived from HDF at passage 10 or HFF at passages 10 and 20 (for OCT4 and NANOG), HFF-CM or HFF-MG (for Ki-67) in hESC or mTeR1 media (for NANOG). The cells were harvested in single-cell format by treatment with trypsin/EDTA and then fixed with ice-cold methanol, blocked by 2 % normal goat serum for 60 min at room temperature, washed, incubated with primary antibody for 1 h at 37 °C, and washed again. Cells were subsequently treated with secondary antibody for 30 min at 37 °C (Ki-67 directly conjugates with FITC), washed, and finally analyzed by flow cytometry. For the negative control, the cells were stained with the appropriate isotype-matched control. Flow cytometric analysis was performed using a FACSCalibur Flow Cytometer (BD Biosciences), and the acquired data were analyzed using BD CellQuest™ Pro software (Ver 0.3.cfab).

Karyotype analysis

For karyotype analysis, the cells were prepared as described by Mollamohammadi et al. (2009).

Gene expression analysis

Total RNA was isolated using the Nucleospin Kit (MN) and treated with a DNaseI RNase-free Kit (Fermentas) in order to remove genomic DNA contamination. We used 2 μg of total RNA for a reverse transcription reaction with the RevertAid First Strand cDNA Synthesis Kit (Fermentas) and a random hexamer primer, according to the manufacturer's instructions. The sequences of primers are presented in supplementary Table 1 (Totonchi et al. 2010).

In vitro differentiation

To demonstrate whether hESC and hiPSC colonies cultured on CM-coated dishes were pluripotent, we assayed the colonies for their ability to differentiate into lineages representative of the three embryonic germ layers, as evidenced by embryoid body (EB) formation and spontaneous differentiation (Mollamohammadi et al. 2009) or direct differentiation to a neuronal lineage (Shahbazi et al. 2011).

Teratoma formation assay

Approximately 2–3 × 10^6 hPSCs that had an undifferentiated morphology were collected by treatment with trypsin/EDTA (1×, Invitrogen, 25300-054), mixed with 40–60 μl MG, and injected into the testes of 5–8-week-old nude mice. Teratomas formed approximately 12 weeks after injection. These teratomas were surgically removed, fixed with Bouin's fixative for 5 days at room temperature, and then embedded in paraffin. Sections were cut at a thickness of 6 μM, processed with hematoxylin and eosin staining, and observed under a bright-field microscope.

All research and animal care procedures were undertaken in strict accordance with the approval of the Institutional Review Board and Institutional Ethical Committee.

Colony formation of single dissociated hESCs and hiPSCs

To evaluate the effect of CM-coated dishes versus MG-coated dishes on the cloning efficiency of single dissociated hESCs and hiPSCs [(number of ALP^+ colonies/number of single dissociated seeded cells) × 100], we analyzed the number of feeder-independent colonies of dissociated single hPSCs.

For this purpose, hESCs and hiPSCs were dissociated into single cells by 0.05 % trypsin/EDTA for 5 min at 37 °C and then collected by gentle pipetting. Cells were seeded at a density of 5 × 10^4/well in six-well plates and allowed to grow. After 7 days, colonies were stained using an ALP kit. Stained colonies were captured using a digital camera (Canon, IXUS 950 IS) and photographs analyzed by ImageJ software, version 1.4.

Cryopreservation of hESCs and hiPSCs

hESCs and hiPSCs were frozen as previously described (Baharvand et al. 2010) with some modifications. Briefly,

hESCs and hiPSCs were trypsinized as single cells and subsequently collected by gentle pipetting. The single dissociated cells were frozen in 10 % dimethyl sulfoxide (DMSO; Sigma-Aldrich, D2650) plus 90 % KOSR, at aliquots of 1×10^6 cells per 250 μL ice-cooled freezing medium. The cryovials were placed in a −80 °C freezer overnight and then transferred into a liquid nitrogen tank the following day for long-term storage.

Fractionation of CM

To determine the effective fraction of CM in undifferentiated growths of hESCs and hiPSCs, we used Millipore's Amicon Ultra-15 centrifugal filter devices (10 K, UFC9 010 08; and 30 K, UFC9 030 08, Millipore) based on the molecular weights of the CM proteins (MWCO). Purification was performed based on the manufacturer's recommendations. Briefly, 12 ml of CM was added to the Amicon Ultra 10 K device and centrifuged at 5,000×*g* (maximum) for approximately 15–60 min. Next, >10- and <10-kDa fractionated CMs were separately coated on culture dishes. The same process was performed by an Amicon Ultra 30 K device for the production of >30- and <30-kDa fractionated CM-coated culture dishes. hESCs (Royan H6, passage 12) were cultured on these four CM fractions for additional analysis.

Statistical analysis

There were nine replications of PE and grading morphology. Approximately 20 clamps were used per replicate in each group (20 clamps/well of a six-well plate). Results were expressed as mean ± standard deviation (SD). The lines were characterized after 11–16 passages in different conditions. Plating, cloning efficiency, and morphology grading were compared using Kruskal–Wallis, Mann–Whitney, or one-way ANOVA followed by Tukey's post hoc test. The PE (based on the ratio of undifferentiated ALK-positive colonies formed per initially seeded hESCs and hiPSCs clumps) was calculated under phase-contrast inverted microscope (CKX 41, Olympus). Cloning efficiency (based on the ratio of ALP-positive colonies formed per initially seeded hESC and hiPSC single cells) was calculated by ImageJ software version 1.4. We repeated the cloning efficiency assay three times. The mean difference was significant at $p < 0.05$.

Results

Preparation of an "optimized CM"

Initially, to evaluate the effect of medium type on the PE of hPSCs, we diluted MG in DMEM/F12 or neurobasal medium. We observed that neurobasal medium was more effective on PE of hPSCs ($p < 0.001$, Fig. 2a). Afterward, the six-well plates were coated for either 5 or 150 min with serum-free neurobasal CMs. The CMs at this step were derived from high- and low-density HDFs (Supplementary Fig. 1) for 24 and 72 h (phase 1, Fig. 1a). High-density-HDF-derived 72-h CM that was coated for 150 min supported the PE and morphology (tightly packed colonies with a high cell nucleus/cytoplasm ratio) of hESCs, in a similar manner as observed for MG (Fig. 2b, at least $p < 0.05$ vs. MG). In comparison with high-density-HDF-derived 24-h CM that had colony rollback and edge detachment, the hESC colonies were more tightly attached (Fig. 2c).

For further optimization of CM, we cultured hESCs in coated plates with pure or half-diluted CM in the presence or absence of ROCKi and simple or layer-by-layer coating (phase 2, Fig. 1a). Although some hESC colonies were attached to diluted CM in the absence of ROCKi, the presence of pure CM and ROCKi in both the coating and layer-by-layer methods for CM coating enabled hESC PE to be comparable with MG without a significant difference (Fig. 2d). Therefore, the high-density-HDF-derived 72-h conditioned neurobasal medium supplemented with ROCKi (1 μM, Y27632) via layer-by-layer coating was used for expansion of hPSCs. We named this in-house prepared ECM "RoGel."

Flow cytometric analysis of NANOG expression as an important stemness factor in hESCs showed the ability of RoGel to sustain hESCs at passages 2 and 32 on CM versus prevalent culture on MG (Fig. 3a, b).

In this condition, hESC proliferation was similar to that of hESCs cultured on MG as detected by flow cytometry for Ki-67, a marker of proliferating cells (Fig. 3c).

Given the role of ECM in cloning efficiency, we then analyzed the number of colonies of dissociated single hPSCs. The numbers of colonies formed after seeding on RoGel were similar compared to MG-coated plates that contained ROCKi (Fig. 3d).

Similar PE results were obtained for Royan H6 hESCs, hiPSC1, and hiPSC4 on RoGel (Fig. 3e). We continued our experiments with either Royan H5 or Royan H6 hESCs.

Effects of source, passage, and age of feeder cells on quality of RoGel

To evaluate the impact of feeder origin and their different passages on stemness of expanded hPSCs on RoGel, we tested the ability of CM derived from HDF (passage 10) and HFF (passages 10 and 20) to support Royan H6 hESCs. Both feeder-derived RoGel at different passages effectively supported OCT4 and NANOG expression of hESCs in a similar manner (Fig. 4a). Further, PE of the

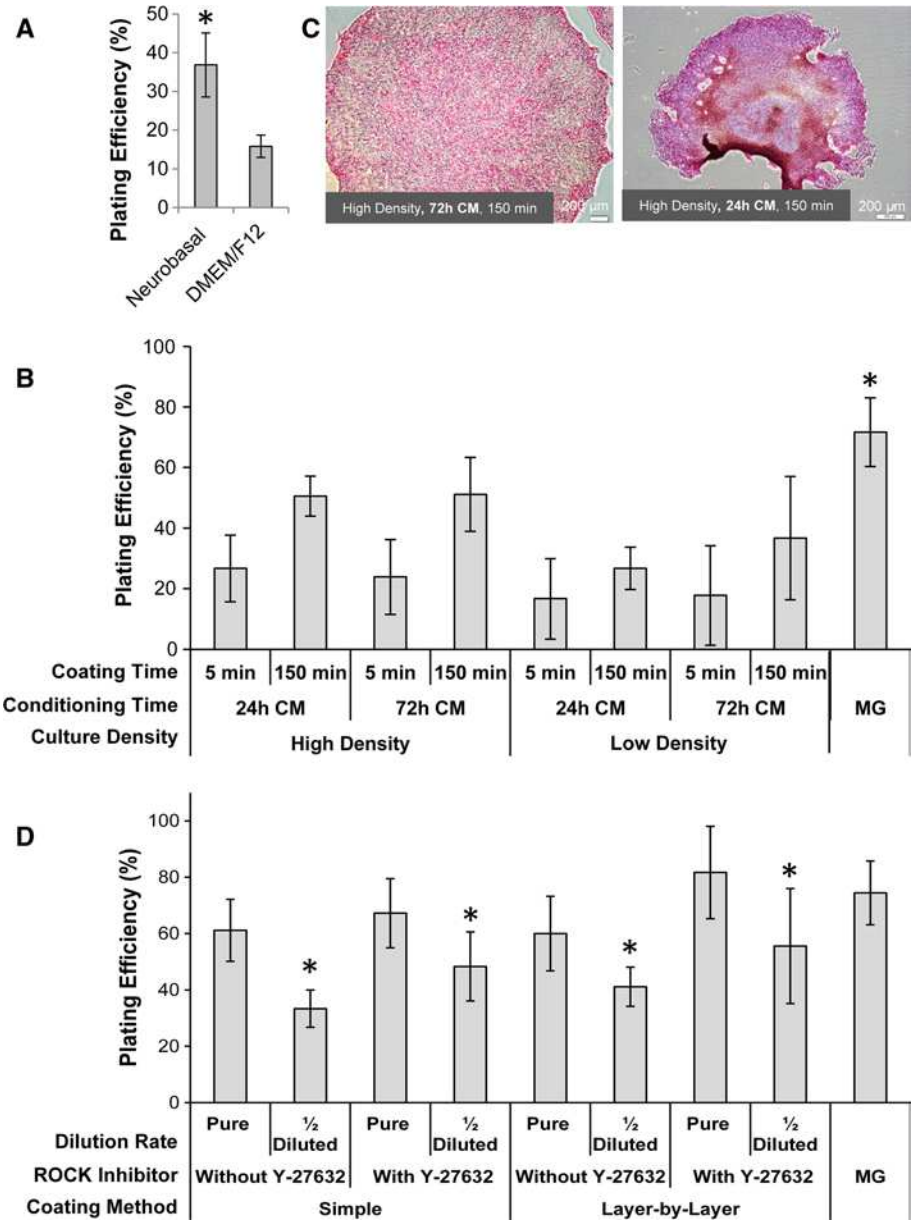

Fig. 2 Plating efficiency (PE) of hPSCs under different conditions to develop a proper substrate. PE calculated as: [(number of ALP-positive colonies/number of seeded explants) × 100]. Conditioned medium (CM) was prepared from serum-free and xeno-free neurobasal medium. Royan H5 hESCs were cultured for 7 days in these conditions. **a** Matrigel (MG) was diluted in DMEM/F12 as a conventional medium for hESC culture or neurobasal medium. The plating efficiency (PE) was significantly higher on MG diluted with neurobasal medium versus DMEM/F12 (*$p < 0.001$). **b** Effect of cell density [high (50,000 cells/cm^2) or low (5,000 cells/cm^2)], time of conditioning (24 and 72 h), and coating (5 or 150 min). High-density, 24-h CM or 72-h CM, and 150-min coating conditions resulted in insignificant PE compared to Matrigel (MG). *Asterisk* at least $p < 0.05$ MG versus other groups. **c** Cells in 72-h CM adhered more tightly to the dish; therefore, we selected these conditions for the next experiment. Colonies were stained with alkaline phosphatase (ALP). **d** Effect of dilution (pure or half) in the presence or absence of Y-27632 (as a ROCK inhibitor factor) in coating CM, and the coating method (simple or layer by layer). The culture of cell clumps in pure CM, presence of Y-276320 in coating CM, and layer-by-layer method of coating resulted in a PE similar to the PE of MG. *Asterisk* at least $p < 0.05$ versus MG

hPSCs was similar on the RoGel of both feeders (Fig. 4b) and CM derived from different passages (10 or 20) when compared with MG (Fig. 4c).

Next, we tested whether CM derived from cultured feeder cells at different times post-passage could influence PE. HDF feeder cells were cultured and CM was prepared after 1, 7, 14, and 28 weeks following preparation of mitomycin C-inactivated HDF. A similar PE was observed until approximately 14-week culture of inactivated HDF (Fig. 4d).

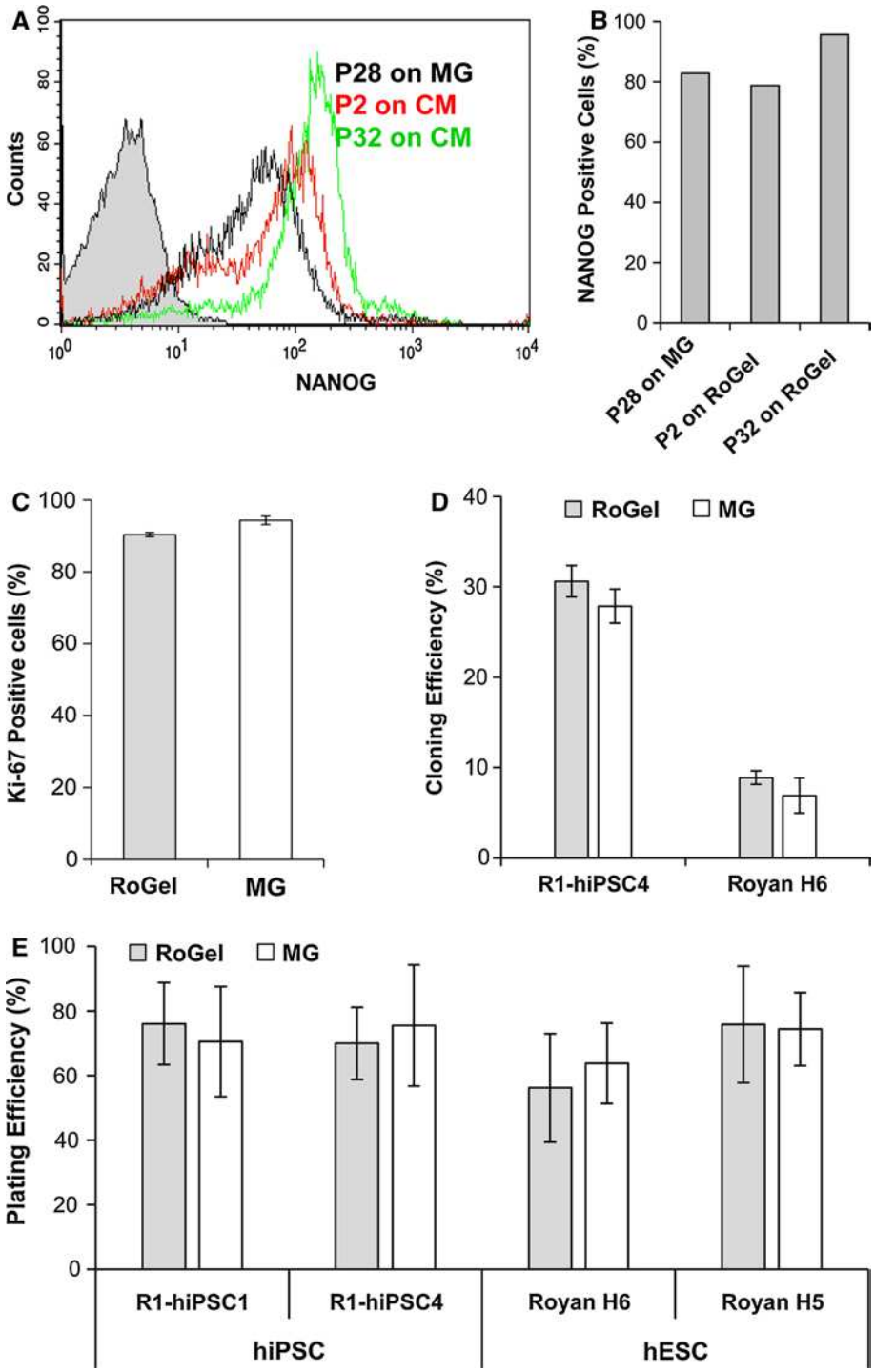

Fig. 3 Behavior of hPSCs on RoGel. **a** Expression of NANOG by flow cytometric analysis during the culture of Royan H6 Passage 28 on MG (*black*) and at low (P2 *red*) and high (P32 *green*) passage number on optimized CM. **b** NANOG-positive cells at different passage on MG and optimized CM. **c** Cellular proliferation on CM and MG indicated similar high Ki67-positive cells. **d** Cloning efficiency [(number of ALP^+ colonies/number of single seeded dissociated cells) × 100] showed results on CM and MG in both Royan H6 hESCs and hiPSC4. **e** The universality of RoGel, neurobasal medium on high-density feeder, 72-h conditioning, 150-min coating, and layer-by-layer coating in the presence of Y-276320, on pure CM, in two hESC, and two hiPSC lines

Effect of temperature and time of CM storage on quality of RoGel

To investigate the effect of temperature maintenance on CM activity, we preserved the CMs at 4 or −20 °C for 1 week, after which the plates were coated. We observed that the PE of Royan H5 hESCs significantly reduced after freezing ($p < 0.001$, Fig. 5). Additionally, the PE decreased significantly when the plates were coated by CM that had been stored at 4 °C for 2 weeks ($p < 0.001$, Fig. 5). To assess the expiration date of RoGel-coated plates, we maintained these plates for 2, 4, 25, 32, 42, and 52 weeks at room temperature in a dark, dry place. We found that plates coated with RoGel were sufficient enough to yield PE; after a year they were still similar to MG control cultures and fresh RoGel (Fig. 5).

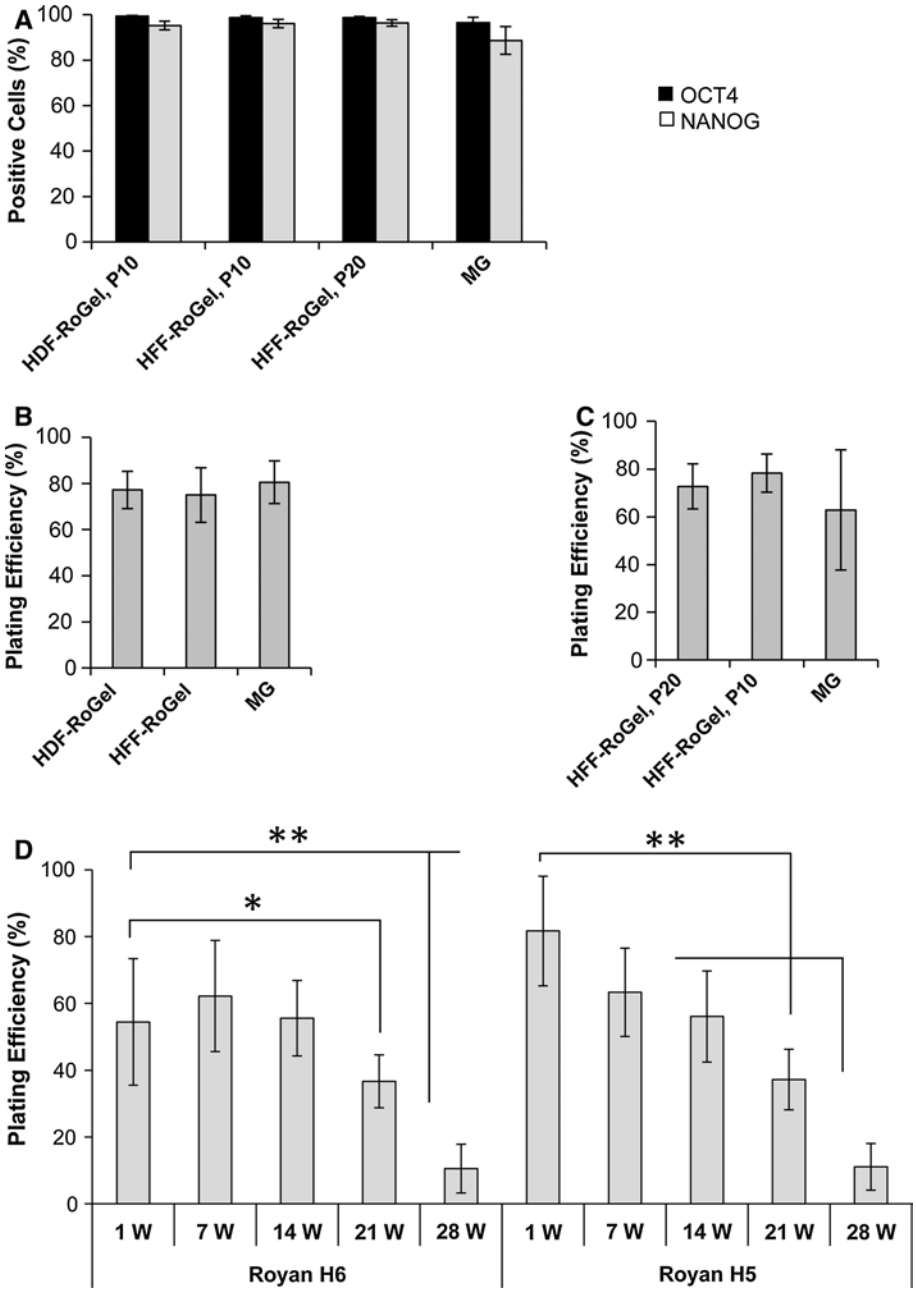

Fig. 4 Influence of source, passage, and age of feeder cells on quality of RoGel. Royan H5 and Royan H6 hESCs were cultured for 7 days in these conditions. The conditioned neurobasal medium on high-density human fibroblast cells for 72 h used as ECM. For hPSC culture, the dishes coated with this pure CM included Y-27632 (800 nM) for 150 min as layer by layer. **a** Flow cytometric analysis of OCT4 and NANOG expressions of Royan H6 at passage numbers 30–32 on MG, RoGel derived from HDF passage 10, and HFF passages 10 and 20 showed no significant differences. The plating efficiency (PE) of Royan H5 on RoGel was not influenced by CM **b** derived from HDF or HFF or **c** their different passages (10 or 20). **d** Effect of age feeder cell culture on RoGel after preparation of inactivated HDF by mitomycin C. Similar PE was observed until about 14-week culture of inactivated HDF. $*p < 0.05$; $**p < 0.001$, 1 W versus the other group

Passaging and freeze/thaw of hPSCs on RoGel

We used numerous types of enzymes to passage hPSCs on RoGel, including trypsin/EDTA, dispase (2 mg/ml; Invitrogen, 17105-041), and StemPro® Accutase® Cell Dissociation Reagent (Invitrogen, A1110-501). Treatment with these enzymes caused colonies to collapse immediately (Fig. 6a, b). In contrast, the colonies were resistant to collagenase type IV (1 mg/ml; Invitrogen, 17104-019), even after 10 min. However, the hESCs and hiPSCs were easily passaged on RoGel-coated plates, just by cutting the colonies into small pieces (close to 0.2 mm^2) by using the keen edge of glass pipettes generated by a flame on the laboratory scale or simply by the use of a STEMPRO® EZPassage™ tool (Invitrogen, 23181-010) in scale-up culture followed by gentle pipetting of colonies on a cold surface (15–20 °C) and replating them on RoGel-coated plates (Fig. 6c–f).

To evaluate the compatibility of RoGel with different culture systems, we tested its ability to support self-renewal of Royan H6 hESCs in both commercially available and chemically defined medium, mTeSR®1 (Stemcell Technologies, 05857), in comparison with the hESC medium-based DMEM/F12 as a control on MG and CM for five to seven

passages. We found efficient support of PE from Royan H6 hESCs in both media (Fig. 7a, b). The cells retained important hESC characteristics, such as doubling time (Fig. 7c) and the specific marker expression, NANOG, as detected by flow cytometry (Fig. 7d).

Next, we assessed whether cells maintained on RoGel could be cryopreserved and then thawed back onto RoGel. For this experiment, we used Royan H6 hESCs that had been maintained for nine passages, cryopreserved, and then thawed back onto RoGel. Recovery was similar to cells maintained on MG and thawed onto MG in terms of both cloning efficiency and ALP expression (Fig. 8).

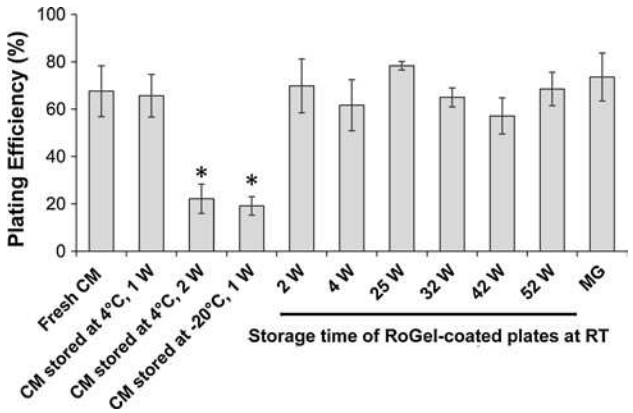

Fig. 5 Influence of temperature and time of CM storage on quality of RoGel. Royan H5 hESCs were cultured for 7 days. Plating efficiency (PE) decreased significantly when RoGel was maintained at 4 °C or freeze/thawing for 2 weeks. Coated plates with RoGel at room temperature (RT) supported PE even after 1 year, which was similar to those of Matrigel (MG) or fresh RoGel. *$p < 0.001$

Characterization of expanded hPSCs on RoGel

Pluripotency maintenance is a critical parameter when evaluating new ECM for hPSC culture. We cultured cells on RoGel for more than ten serial passages in hESC medium. CM and hPSC marker expression were evaluated at the end of each passage. Typical hPSC colony morphology for cells grown on CM was similar to that of MG cultures (Supplementary Fig. 2A). Immunofluorescence evaluation of the hPSC-specific markers OCT4, SSEA3, and TRA-1-81 showed expressions of markers cultured on RoGel and MG control cultures (Supplementary Fig. 2A). hPSCs also expressed NANOG, SOX2, and KLF4 as detected by RT-PCR (data not shown). Karyotype analysis was performed in hESCs and hiPSCs that were propagated on RoGel to demonstrate the absence of chromosomal abnormalities. The results showed normal karyotype for both hESC and hiPSC lines (Supplementary Fig. 2B).

The pluripotency of hPSCs maintained on RoGel was also assessed by in vitro spontaneous and directed differentiation of EBs. Tissue components that expressed markers of the three germ layers, such as *FOXA2* and *ALB* for the endoderm, *PAX6* and *Nestin* for the ectoderm, and *Brachyury* and *GATA4* for the mesoderm, were all detected by RT-PCR (Supplementary Fig. 3A). TUJ1 (β-tubulin III), as a

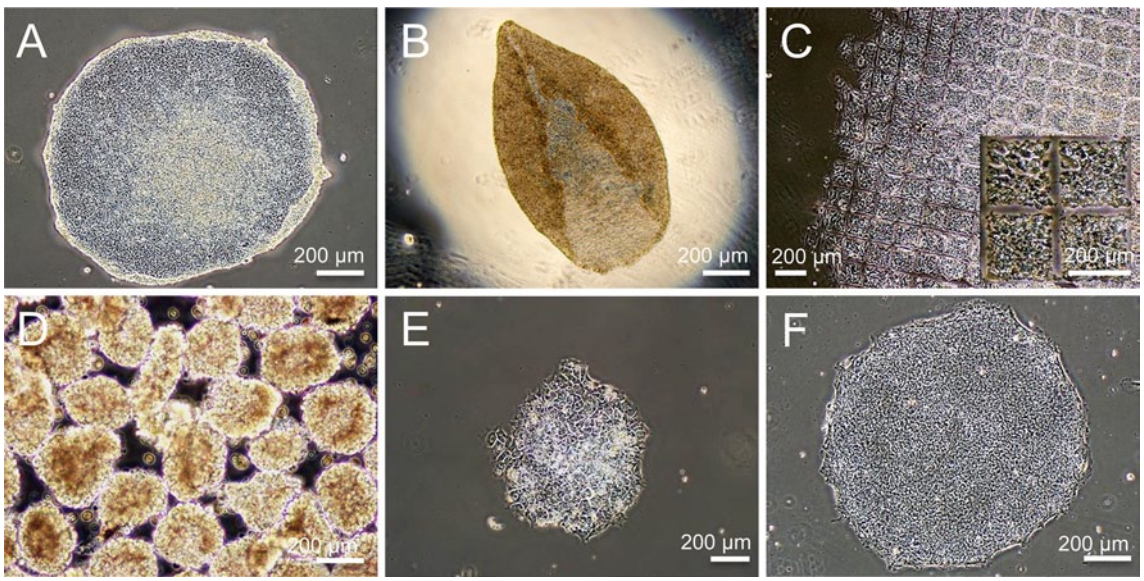

Fig. 6 Passaging of hPSCs on RoGel. **a**, **b** Attachment of Royan H5 colonies onto RoGel-coated plates was weak and sensitive to enzymatic passaging. After enzymatic treatment, whole colonies immediately detached. **c**–**f** Colonies were passaged mechanically either by a flamed processed Pasteur pipette in laboratory scale or STEMPRO® EZPassage™ tool in large-scale passaging followed by gentle pipetting and replating on RoGel-coated plates. **c** A colony that cut by STEMPRO® EZPassage™ tool. **d** Colony pieces ready for transfer to the new vessels. **e**, **f** Part of an hESC colony at 2 and 7 days after culture on RoGel

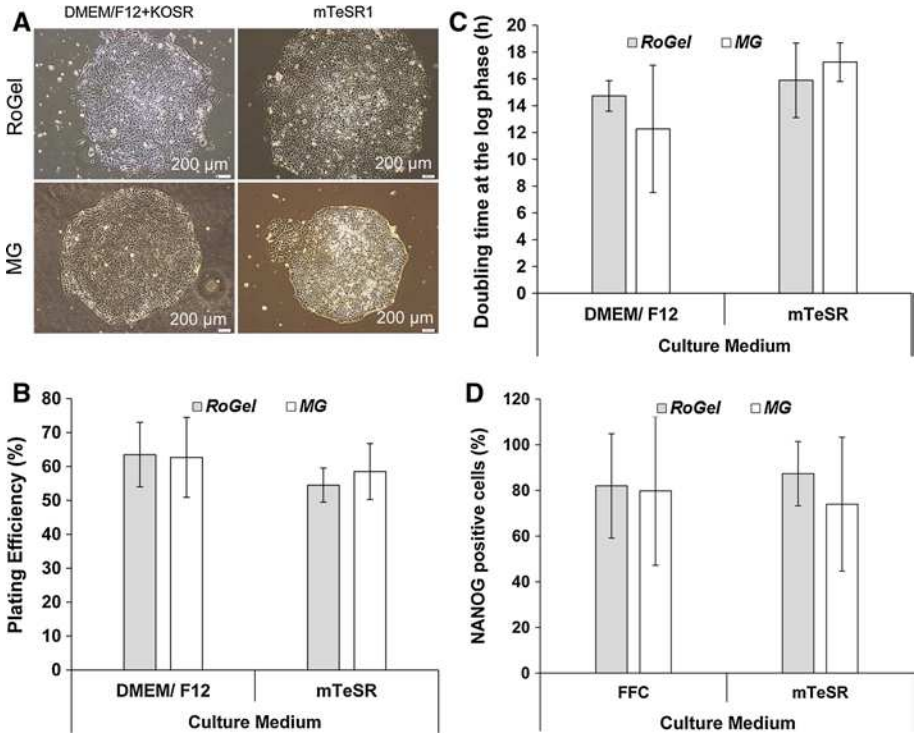

Fig. 7 Effect of medium on compatibility of RoGel. Royan H6 hESCs were cultured for 7 days. **a** hPSCs were maintained for at least five passages on MG and RoGel in DMEM/F12 and mTeSR1 media. **b** Cells showed similar plating efficiency (PE), **c** doubling time population, and **d** expression of stemness-specific marker, NANOG

mature neuronal marker, was observed by immunocytofluorescence (Supplementary Fig. 3B). Finally, we examined whether the pluripotency of hPSC (Royan H6 and hiPSC4) cells was retained after seven and 12 passages in hESC medium on CM-coated dishes by following their ability to generate teratomas in nude mice. Teratomas formed 12 weeks after transplantation, and histological analyses revealed the presence of cells from all three germ layers (Supplementary Fig. 3C).

Fraction of >30-kDa RoGel supported self-renewal of hPSCs

To assess the effective fraction of CM in the undifferentiated growth of hESCs and hiPSCs, we used >10-, <10-, >30-, and <30-kDa fractionated RoGel.

The plates were separately coated by fractionated CM. Royan H6 hESCs were cultured on the four fractions. Analysis of colony morphology showed that cells grew with hESC morphology when the plates were coated with >10- and >30-kDa fractionated RoGel. We found that <10 and <30 kDa that contained 10-kDa molecules were not suitable; thus, we concluded that molecules of >30 kDa were more important (Supplementary Fig. 4).

Then, we focused on the shapes of coated surfaces by MG, simple or layer-by-layer RoGel, and fractionated RoGel. We found that the air-dried coated surfaces of RoGel with >10- and >30-kDa fractions were similar to those of air-dried MG and whole RoGel (Supplementary Fig. 5).

Discussion

Several studies have replaced MG with chemically (Melkoumian et al. 2010; Villa-Diaz et al. 2010) or biologically modified surfaces (Rodin et al. 2010; Klim et al. 2010; Ludwig et al. 2006) to culture hPSCs. These studies have reported cell adhesion, long-term self-renewal, pluripotency, and karyotypic stability of hESCs when cultured on a chemical- or peptide-coated surface. There are some drawbacks to these biological materials, such as limited scalability, lack of availability in some laboratories, high batch-to-batch variability, and the cost of routine culture [for review see (Villa-Diaz et al. 2013)]. In addition, animal-derived materials have xenogeneic contamination; most of the ECMs were generally effective for short-term propagation. Researchers still seek xeno-free culture plate coatings that do not induce cellular differentiation in hPSCs and save chromosomal integrity (Draper et al. 2004; Ludwig et al. 2006). In this study, we reported the development of a simple, less intensive and time-consuming, in-house-prepared, and low-cost ECM for the expansion of hESCs and hiPSCs. Moreover, the coated plates could be simply preserved at room temperature for long periods of time. Our results have demonstrated that hPSCs cultured on RoGel maintained stable, typical hESC morphology, proliferation, expression of stem cell markers, in vitro pluripotency, and normal karyotype.

We prepared our RoGel from either HDF or HFF with neurobasal medium without any serum or KOSR.

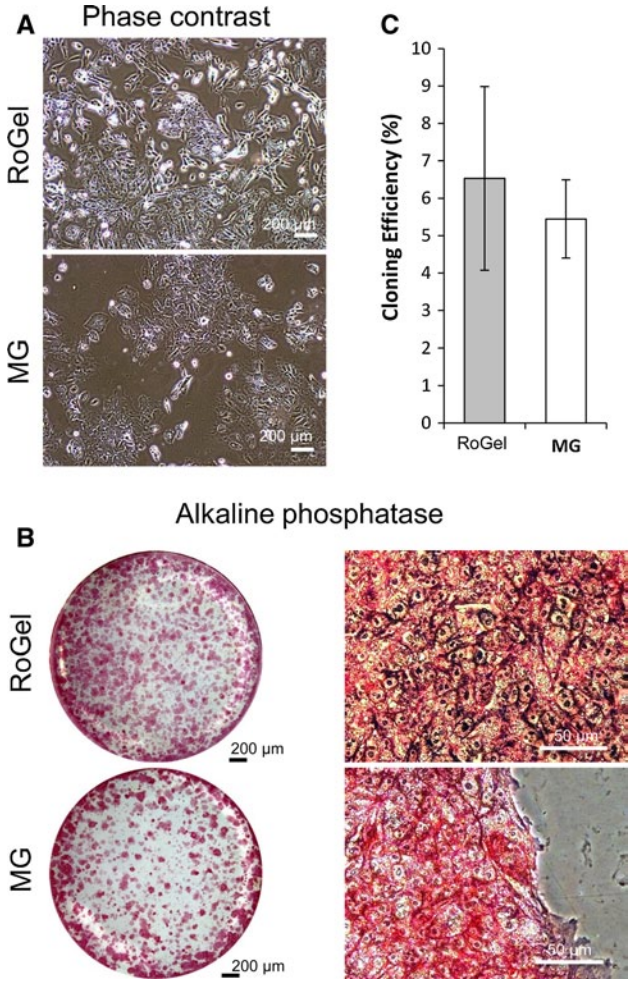

Fig. 8 Freeze/thaw of hPSCs on RoGel. Representative photograph of hESCs (Royan H6), **a** phase-contrast and **b** ALP-positive staining on RoGel and Matrigel (MG). **c** Evaluation of cloning efficiency for hPSCs. The cloning efficiency was analyzed on the basis of the ratio of ALP-positive colonies formed per initially seeded hESC by ImageJ software

The efficient impact of neurobasal medium on the maintenance of hPSCs on a suspension culture has been previously described (Steiner et al. 2010). Other research groups subsequently applied this medium to expand hPSCs in large-scale suspension culture systems as effective basal medium (Larijani et al. 2011).

We added ROCKi in CM because we discovered earlier that the addition of ROCKi to ECM increased plating (1.5- to twofold) and cloning efficiencies significantly when it was added to medium (Pakzad et al. 2010; Mollamohammadi et al. 2009) compared to those that contained ROCKi in a culture medium only. We added ROCKi at a very low concentration (1 μM) in RoGel, because similar plating efficiencies were observed using a concentration of Y-27632 (800 nM = EC50; Pakzad et al. 2010) when compared to a higher concentration of Y-27632 (10 μM; Watanabe et al. 2007).

Simple mechanical passaging of the cells on a cold surface provides long-term maintenance of the cells with a stable karyotype. This potential of RoGel resulted in scale-up culture of hPSCs, just by gently pipetting colonies on a cold surface (15–20 °C) and replating on RoGel-coated plates. The essence of cell attachment on RoGel is the same of recently produced thermoresponsive hydrogel (Zhang et al. 2013).

Since we show that the origin and passage number of fibroblast cells have not negative effect on quality of RoGel, easily it can be used in research and clinical laboratories for each of the patients who need safe pluripotent stem cell culture.

This human-feeder-derived CM decreases the risk of cellular contamination and animal-derived pathogens. It also provides a scalable and repeatable platform for the clinical grade culture of hPSCs. HDF- and HFF-derived CMs and even high-passage (passage 20)-feeder-derived CMs have a similar effect on hPSC expansion. We have shown that long-term culture of feeders does not influence the quality of CM in support of hPSC self-renewal.

This research has also shown that there is suitable compatibility with other commercially available chemically defined but not xeno-free media, mTeSR1, and xeno-free but undefined KOSR-supplemented medium for the expansion of hPSCs on RoGel. In addition, we demonstrated that hPSCs cultured on RoGel could be cryopreserved and successfully thawed back onto RoGel for further expansion or differentiation, which is important for the production of clinical grade hPSC banks. Notably, we have demonstrated that RoGel provides a scalable, enzyme-free passage of hPSCs that is important to maintain a stable cell karyotype (Draper et al. 2004). Finally, in contrast to UV-treated vessels (Iwasa et al. 2011), hPSCs maintained self-renewal on plates preserved for a long term (1 year) at room temperature. This property is important for the distribution of coated plates and the expansion of scalable hPSCs. hPSCs grew when the plates were coated with >30-kDa fractionated RoGel. Thus, the secreted ECM molecules, such as all types of collagens, laminin, and its isoforms, fibronectin, lumican, periostin, and heparan sulfate proteoglycan (Rodin et al. 2010; Domogatskaya et al. 2008; Prowse et al. 2005; Abraham et al. 2010), were important in secretion, and growth factors and other secreted proteins with low molecular weight (<30 kDa) were not important in this regard.

ECM molecules are large, complex, and often multimeric structures. Their recombinant production is generally difficult or not cost-effective (Ruggiero and Koch 2008). Meanwhile, ECM is a natural mixture of molecules; hence, CM-based RoGel mimics natural ECM. Several research groups have shown the proteome of CM of mouse and human fibroblast feeders in order to replace its critical

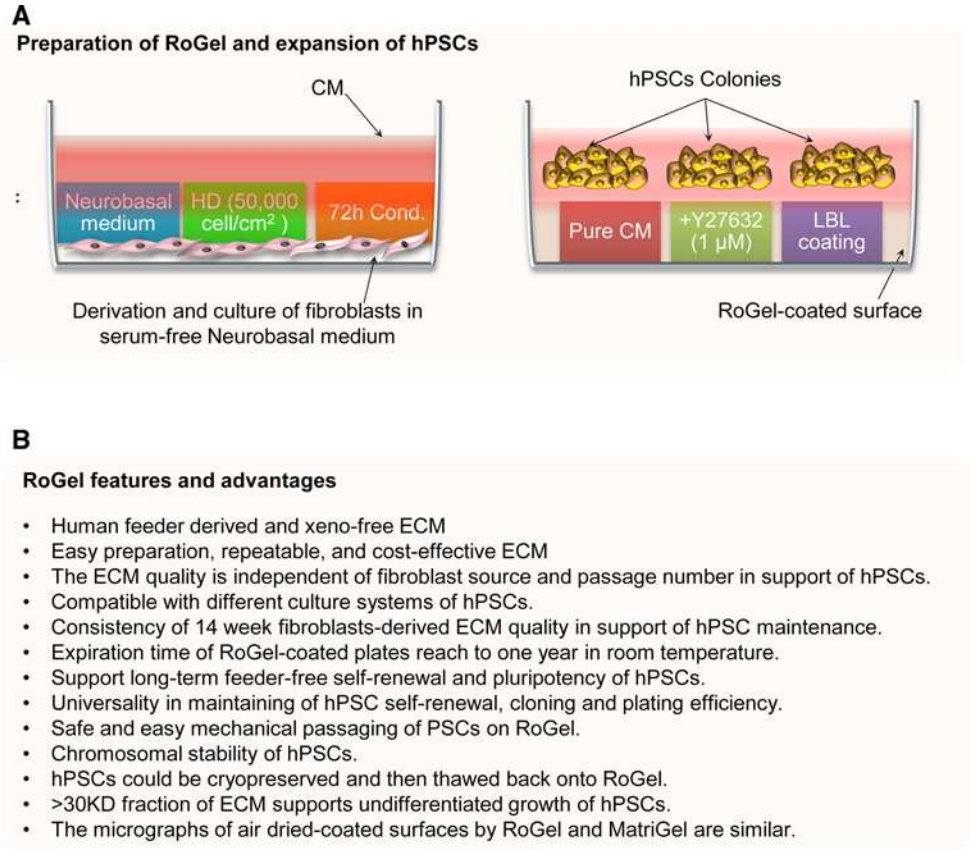

Fig. 9 Optimized conditions for RoGel production and its features and advantages. **a** Optimized CM as RoGel ECM. RoGel was prepared by neurobasal medium conditioned on mitomycin-inactivated high-density (HD) human fibroblasts (50,000 cells/cm^2) for 72 h. Next, undiluted CM supplemented with 1 μM ROCKi (Y27632) and used for coating via layer-by-layer method (**a**). **b** Features and advantages of RoGel

molecule(s) to establish a defined condition culture system for hPSCs (Gonzalez et al. 2010; Prowse et al. 2005, 2007; Lim and Bodnar 2002; Chin et al. 2007). Recently, it has been demonstrated that HDF secretes laminin-511 (Hongisto et al. 2012) and expresses the laminin-binding integrins α3β1, α6β1, and α7β1. They have a critical role in the maintenance of hPSCs (Vuoristo et al. 2009; Rodin et al. 2010; Miyazaki et al. 2008; Evseenko et al. 2009; Domogatskaya et al. 2008). Other efficient ECM molecules such as vitronectin (Braam et al. 2008; Prowse et al. 2010) and the recently reported E-cadherin type I (Li et al. 2010; Xu et al. 2010) have a molecular weight of >30 kDa.

Conclusion

In summary, we have developed a simple, repeatable, in-house-prepared, cost-effective, and xeno-free ECM designated here as RoGel for the maintenance of hPSC self-renewal. RoGel was prepared by neurobasal medium supplemented with 2 mM L-Gln, 0.1 mM β-ME, 1 % NEAAs, and 1 % ITS and then conditioned for 72 h on mitomycin-inactivated high-density human fibroblasts (50,000 cells/cm^2). The undiluted CM supplemented with 1 μM ROCKi (Y27632) was used to coat plates via the layer-by-layer method (Fig. 9a). RoGel has numerous advantages in comparison with Matrigel (Fig. 9b). This ECM has the potential for the expansion of hPSCs and directed differentiation in place of MG. We believe that this ECM will be useful for both research purposes and future therapeutic applications.

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Conflict of interest The authors have no conflict of interest to declare.

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