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Human iPS cell-derived fibroblast-like cells as feeder layers for iPS cell derivation and expansion

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Mouse embryonic fibroblasts (MEFs) are commonly used as feeder cells for the generation of human induced pluripotent stem cells (hiPSCs). However, medical applications of cell derivatives of hiPSCs generated with a MEF feeder system run the risk of having xeno-factor contamination due to long-term cell culturing under an animal factor-containing environment. We developed a new method for the derivation of human fibroblast-like cells (FLCs) from a previously established hiPSC line in an FLC differentiation medium. The method was based on direct differentiation of hiPSCs seeded on Matrigel followed by expansion of differentiating cells on gelatin. Using inactivated FLCs as feeder layers, primary human foreskin fibroblasts were successfully reprogrammed into a state of pluripotency by Oct4, Sox2 Klf4, and c-Myc (OSKM) transcription factor genes, with a reprogramming efficiency under an optimized condition superior to that obtained on MEF feeder layers. Furthermore, the FLCs were more effective in supporting the growth of human pluripotent stem cells. The pluripotency and differentiation capability of the cells cultured on FLC feeder layers were well retained. Our results suggest that FLCs are a safe alternative to MEFs for hiPSC generation and expansion, especially in the clinical settings wherein hiPSC derivatives will be used for medical treatment.

[**Key words:** Induced pluripotent stem cells; Fibroblast-like cells; Cell reprogramming]

The advances in human induced pluripotent stem cell (hiPSC) research have opened the door to clinically apply human cells in regenerative medicine. Many of currently tested hiPSCs were generated on mouse embryonic fibroblast (MEF)-based feeder layers, which permit the propagation and maintenance of undifferentiated cells. However, the potential risks of xeno-factor contamination and animal pathogen transmission are the major concerns for hiPSCs generated on MEFs and could be serious hurdles in obtaining regulatory approval for clinical applications of hiPSC derivatives (1). Although there is no report of xeno-factor contamination in hiPSCs to date, such contamination has been observed in human embryonic stem cells (hESCs). The expression of xeno-factor sialic acid *N*-glycolylneuraminic acid (Neu5Gc) was detected on the surface of hESCs after the cells were cultured on MEFs for a long period. This raises the clinical concern over immunogenic responses after the injection of hESCs or their progeny into the recipient (2).

In order to eliminate xeno-factor contamination, many research groups have generated hiPSCs by using the parental cells as feeder layers, like human fibroblast cells (1), adipose cells (3), and amniocytes (4). While using parental cells as feeder layers avoids xeno-factor contamination, insufficient numbers of cells available for large-scale and long-term process could hinder the expansion and maintenance of the generated hiPSCs for therapeutic applications.

Feeder-free, extracellular matrix (ECM)- or synthetic substrate-based cultivation has also been extensively studied to produce and maintain hiPSCs. However, the reprogramming efficiencies to derive hiPSCs with these cultivation systems remain low (3,5–8). Karyotypic abnormality is another concern associated with long-term maintenance of human pluripotent stem cells on a feeder-free system. Related to this, genomic instability has been reported for hESCs after long-term culture on Matrigel (9).

The present study was attempted to develop human cell-based feeder systems without xeno-components. We tested whether human fibroblast-like cells (FLCs) derived from previously established hiPSCs could serve as an alternative feeder layer to MEFs.

MATERIALS AND METHODS

FLC derivation from hiPSCs A hiPSC clone 5.9 generated from human foreskin fibroblasts (HFFs) in one of our previous studies (10) was used for FLC derivation. hiPSCs were maintained on Matrigel (BD, Franklin Lakes, NJ, USA) and subcultured every 7 days by enzyme digestion with 1 mg/ml Dispase (StemCell Technologies, Vancouver, BC, Canada) at 37°C for 5–7 min. The mTeSR1 culture medium (StemCell Technologies) was changed daily. For FLC derivation, hiPSCs were treated with three different protocols in a standard FLC medium composed of DMEM-high glucose (Thermo Scientific, Waltham, MA, USA), 10% FBS (Thermo Scientific) and 0.1 mM nonessential amino acids (NEAA) (Invitrogen, Carlsbad, CA, USA).

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Protocol A: hiPSCs were detached by 0.1 mg/ml Dispase digestion at 37°C for 20–30 min, and transferred to six-well ultra-low attachment plate (Corning, Corning, NY) for embryonic body (EB) formation. EBs were cultured in medium consisting of 80% DMEM/F12 (Invitrogen), 20% KnockOut Replacement Serum (Invitrogen), 2 mM L-glutamine (PAN biotech, GER), 0.1 mM β-Mercaptoethanol (Sigma–Aldrich, St. Louis, MO, USA), 0.1 mM NEAA, and penicillin/streptomycin (PAN biotech) (11). After culture for 5 days, EBs were transferred to 0.1% gelatin pre-coated six-well plate and cultured in the FLC medium. The outgrowing single cells from attached EBs were maintained in the gelatin-coated plate and subcultured on reaching confluence. The FLC medium was changed every 2–3 days and confluent cells were passaged with 0.05% Trypsin-EDTA (Invitrogen).

Protocol B: hiPSC colonies were digested with 1 mg/ml Dispase at 37°C for 5–7 min, then manually detached and transferred to 0.1% gelatin-coated six-well plates. The colonies were allowed to differentiate in the FLC medium as described in protocol A.

Protocol C: After hiPSCs cultured on Matrigel with the mTeSR1 medium reached 80% confluence, the medium was removed and the cells were washed with 1× PBS twice. The FLC medium was added to allow direct differentiation of hiPSCs. The culture medium was refreshed daily during the first 4 days, and changed every 2 days for 6 to 12 more days. Cells were then digested with 0.05% Trypsin-EDTA and plated into Matrigel-coated 6-well plate at a density of 1×10^6 per well. After cells reached confluence (usually for 5 days) on the Matrigel-coated plates, they were digested and seeded into 0.1% gelatin-coated six-well plates at a density of 2×10^5 per well. Cells became homogenous and displayed fibroblast-like morphology after culturing on gelatin-coated plates for 3–5 days. The derived FLCs were subcultured every 3–4 days. They can be cryopreserved in liquid nitrogen, with a freezing medium consisting of the FLC medium supplemented with 10% DMSO (Sigma–Aldrich).

Cellular phenotyping of hiPSC-derived FLCs For RT-PCR analysis, total RNA was extracted from cultured cells using RNeasy kit (Qiagen, Chatsworth, CA, USA). In order to eliminate genomic DNA contamination, the extracted RNA was treated with DNase I (Qiagen) for 10 min at room temperature and further purified with RNeasy column. Final RNA samples were re-suspended in RNase-free water and the quality of RNA was examined by NanoDrop 1000 spectrophotometer (Thermo Scientific). One microgram RNA in 20 μL reaction volume was reverse transcribed into the first strand cDNA with the Super ScriptTM III Reverse Transcriptase kit (Invitrogen) (RT+), while the negative control (RT-) was treated in the same way without the addition of SuperScript III Reverse Transcriptase. The obtained cDNA (1 μl out of 20 μl) was amplified with TaqMan Fast Universal PCR Master Mix (Invitrogen). The PCR condition was performed as the following process: initial 95°C for 10 s, then 35 cycles by 95°C for 1 s, 62°C for 25 s, and extension 72°C for 1 min. Human housekeeping gene GAPDH was selected as an internal control and the PCR products were analyzed by 1.5% agarose gels.

For flow-cytometry analysis of surface antigen expression, antibodies against CD44, CD90, CD71 and CD31 (PECAM-1, Abcam, USA) and IgG1 isotype control were used. Briefly, hiPSCs and FLCs were dissociated into single cells with 0.05% trypsin-EDTA for 3 min, and then the cells were harvested for antibody staining. After incubation for 30 min at 4°C in the dark, cells were washed with staining buffer (PBS + 1% FBS) for three times. For flow cytometer analysis, at least 1×10^4 cells were gated. Flow-cytometry data were analyzed with software Kaluza.

Reprogramming of HFFs on hiPSC-derived FLCs To use hiPSC-derived FLCs as feeder cells for cell reprogramming, these cells were inactivated by 10 mg/ml mitomycin C (Millipore) for 3 h. A method facilitating site-specific integration of the OSKM genes was used for reprogramming of HFFs (Millipore) on hiPSC-derived FLCs. The method employed baculoviral transduction-based zinc finger nuclease (ZFN) technology to deliver ZFNs and an OSKM gene-containing ZFN donor into the AAVS1 locus of HFFs. Briefly, 1×10^4 HFFs cultured in FibroGRO-LS Complete Media (Millipore) were co-transduced with baculoviral vectors (BVs) carrying ZFNs and the ZFN donor twice at day 1 and day 8. The used titers of BV-ZFN and of BV-donor were 100 and 50 multiplicity of infection (MOI) per cell, respectively. After drug selection with 200 μg/ml G418 for 10 days, surviving cells were placed onto FLCs for colony formation. MEFs and Matrigel-coated plates were also included for comparison. An hiPSC medium (10) was used for cells plated on FLCs and MEFs while the mTeSR1 medium was used for cells cultured on Matrigel. Culture medium was changed very other day, and embryonic stem cell (ESC)-like colonies began to appear 7 days after cell seeding. To evaluate the reprogramming efficiency, ESC-like colonies were stained with Alkaline Phosphatase (AP) staining kit (Millipore), and the number of pink color-stained colonies was manually counted.

Characterization of hiPSCs newly generated on FLCs To detect *AAVS1* site specific transgene integration by genome DNA PCR, genomic DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen). The same primers and PCR condition as previously reported were used to detect four factor integration events in AAVS1 site (12). KAPA HiFi Hotstart Readymix (KAPA Biosystem, Woburn, MA, USA) was used for amplification. The cycling conditions were as follows: 95°C for 5 min, 35 cycles by 98°C for 20 s, and 75°C for 15 s and 72°C for 90 s, extension by 72°C for 5 min. PCR products were analyzed with 1% agarose gel.

hiPSCs were also characterized by real-time PCR quantification of pluripotent markers. For real-time PCR, cDNA (0.1 μl out of 20 μl) was amplified using the iQ SYBR Green Supermix (10 μl) and PCR product was detected under the iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The procedure of PCR program was: initial 95°C for 3 min, then 40 cycles by 95°C for 15 s and 60°C for 1 min. In the experiment, each sample had three biological repeats and each biological repeat had triplicates for each targeted gene. The real-time PCR data was analyzed with software DataAssist V3.0 (Invitrogen). Human housekeeping gene ACTB and B2M were selected as internal control for normalization, and gene expression level was then calculated using the **2**-ΔΔCt method.

Characterization of effects of hiPSC-derived FLCs on pluripotent stem cells To evaluate supportive effects of hiPSC-derived FLCs on human pluripotent stem cell growth, the colony growth rates of a newly generated hiPSC clone and H1 human ESC line were determined by measuring the daily increase in colony diameter. These clones were maintained either on FLCs or MEFs for at least 5 passages before evaluation. For the evaluation, ten colonies of each pluripotent stem cell clones were used for photograph. The diameter of the colonies was measured from images that were taken on day 2 and day 5 after cell seeding. The colony growth rate was calculated according to the formula:

$$\frac{\text{Diameter of day 5} - \text{Diameter of day 2}}{\text{3 days}} \quad (1)$$

Expression levels of pluripotent stem cell markers in these colonies were quantified by qPCR. Expression levels of the markers in the colonies cultured on Matrigel/mTeSR1 were used as internal controls.

To evaluate effects of hiPSC-derived FLCs on differentiation capability of human pluripotent stem cells, a newly generated hiPSC clone and H1 human ESC line were used for EB formation after the clones were maintained either on FLCs or MEFs for at least 5 passages. EBs were formed as described above and used for qPCR analysis.

To test the teratoma formation ability of hiPSCs, 1×10^6 cells were dissociated using Accutase (Millipore) and mixed with 0.5×10^6 mitomycin-treated HFF in PBS to a total volume of 50 μl. Prior to transplantation, 50 μl undiluted cold Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA) was added to the prepared cells. The cells were injected into the rear legs of 5-week-old nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice. Two months after injection the resulting teratomas were removed and fixed in 4% paraformaldehyde, embedded in paraffin, cut in 5-μm sections, and stained with hematoxylin and eosin. All handling and care of animals was performed according to the guidelines for the Care and Use of Animals for Scientific Purposes issued by the National Advisory Committee for Laboratory Animal Research, Singapore.

Statistical analysis The statistical significance of differences was determined by unpaired Student's *t*-test and ANOVA. *P*-value of <0.05 was considered to be statistically significant.

RESULTS

Establishing a new method for FLC derivation from hiPSCs Previous studies have reported FLC derivation from hESCs by EB-mediated differentiation (13–15). We first tested this method for FLC derivation from hiPSCs. The hiPSC clone 5.9, a hiPSC clone established using a polycistronic lentiviral vector in our lab previously (10) was used. The hiPSC clone had been passaged at weekly intervals and was in excess of 60 serial passages when used for FLC derivation. We employed a standard method (protocol A) to form hiPSC-EBs and then placed them into gelatin-coated plates with an FLC differentiation medium reported by Chen et al. (16). Single cell began to outgrow from EBs next day and formed radial monolayers around EBs in approximately 5 days. Fibroblast-like cells could be observed on day 7. They proliferated slowly and it took 4–5 weeks for fibroblast-like cells expanded into a homogenous population (Fig. 1A). We also tried the differentiation of hiPSC colonies after transferring them directly to gelatin-coated plates without EB formation (protocol B) (Fig. 1B). hiPSCs started differentiation immediately after the transfer, although severe cell death was observed in the first several days. However, similar to the EB formation method, the overall yield of FLCs was low and it also took 4–5 weeks to generate a morphologically homogenous population.

In the third method (Fig. 1C), we allowed the direct differentiation of hiPSC colonies in Matrigel-coated plates first by replacing the mTeSR1 medium with the FLC differentiation medium. Differentiated cells expanded rapidly to cover the whole culture plate,