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Nanofibrous gelatin substrates for long-term expansion of human pluripotent stem cells

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ABSTRACT

Nanofibrous gelatin substrates are suited for long-term expansion of human pluripotent stem cells (hPSCs) under feeder- and serum-free culture conditions. A combinatorial library with different sets of processing parameters was established to assess the culture performance of hPSCs on nanofibrous substrates in terms of cell adhesion and growth rate, using Matrigel as control. Then, the optimal conditions were applied to long-term expansion of hPSCs with several cell lines, showing a maintained pluripotency over more than 20 passages without introducing any abnormal chromosome. In addition, this approach allowed us to avoid enzymatic disassociation and mechanic cutting during passages, thereby promoting a better hPSC culture and long-term expansion.

1. Introduction

In cell biology, glass slides, plastic dishes, flasks and multi-wells are extensively used for *in vitro* assays. The addition of biophysical and/or biochemical cues on culture surfaces can easily modulate cell functions, e.g., cell adhesion, migration, growth and apoptosis. However, higher functions such as fate decision of human pluripotent stem cells (hPSCs) are still challenging to regulate. hPSCs, including both human embryonic stem cells (hESCs) [1] and human induced pluripotent stem cells (hiPSCs) [2], hold high potential for a large variety of applications due to their unique capability of infinitive self-renewal and controlled differentiation to different types of target cells [3]. Under conventional culture conditions, hPSCs are impure [4,5] and show systematically chromosomal abnormalities [6] and high tumorigenic potential [7,8]. To achieve a clinic stage with good manufacturing practices (GMP) [9], great efforts have been devoted to the development of new growth medium and new extracellular support as well as other critical environmental factors. As expected, serum-free and chemically defined media such as mTeSR1 [10,11] and E8 medium [12] are now commercially available. In contrast, the development of extracellular supports or advanced culture substrates has undergone a dynamic change. Originally, mitotically inactivated mouse embryonic fibroblasts (MEFs) have been used as feeder cells to co-culture hPSCs for their maintenance. Although this approach allows routine propagation of hPSCs, it is not suited for clinic purposes because of the risk of cross-transfer of animal pathogens. Matrigel, a commercial product of protein mixture extracted from mouse Engelbreth–Holm–Swarm tumor, is now widely used for surface coating of hPSC culture substrate but it also contains unknown animal factors. Consequently, recombinant proteins such as vitronectin [13] and laminin [14–17] as well as synthetic polymers [18] and peptide-acrylate composites [19] have been proposed as surface coating substances. These components are in general well-defined but high-cost. Surprisingly, collagen, which is the most important and abundant extracellular component, has not been studied for hPSC self-renewal. Collagen as well as its hydrolyzed product, gelatin, are rich in arginine-glycine-aspartate (RGD) peptide sequences that are required for integrin-mediated cell adhesion and growth via activation of cellular signaling pathways. Also

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both can be manufactured at low cost for cosmetic, food or medical uses with the general requirement of GMP. In particular, gelatin can be highly purified and easily processed. However, hPSCs cannot propagate on gelatin-coated substrates, showing a low survival capacity and rapid loss of the pluripotency after few passages. One possible alternation is to introduce nanofibrous morphology to the gelatin coating surfaces in favor of hPSC expansion. Indeed, so far the most successful studies of hPSC expansion were based on flat surface cultures with appropriate coatings, but it is generally believed that nanofibrous substrates would support pluripotency maintenance of stem cells. The advantage of using nanofibrous substrates in cell culture has already been shown previously with a number of synthetic polymers and a few hPSC passages [20–23]. Intuitively, the surface morphology of nanofibrous substrates resembles some parts of the *in vivo* extracellular matrices, generally showing nanoscaled textures with porous features. This particular type of surface roughness may facilitate the diffusional transport of cytokines and large proteins and possibly the cell traction due to increased or decreased ligand accessibility, thereby allowing a more efficient coordination of adhesion-growth multi-pathway signalings.

The purpose of this work is to develop and assess the potential of nanofibrous gelatin substrates for long-term expansion of hPSCs. Electrospinning, which is a robust and straightforward manufacturing method, has been used to produce gelatin nanofibers with different diameters. We first evaluated the adhesion efficiency and growth rate of hPSCs on nanofibrous substrates prepared using solutions of different gelatin molecular weights and concentrations, different solvent compositions and different electrospinning times, with Matrigel as control. Then, we examined the hPSC culture performance by varying the cross-linking reagents and times of the gelatin nanofibers. To simplify our assessment, we selected nanofibrous substrates which showed the same performance as Matrigel, in term of hPSC adhesion efficiency at 4 h after seeding and hPSC doubling time at day 4 for long-term expansion assays. Up to 20 passages have been tested for several cell lines, all showing undifferentiated status and no abnormal karyotype changes. Finally, the gene expression patterns of integrin family members were determined in comparison to that of Matrigel, providing additional evidence of the suitability of nanofibrous gelatin substrates for long-term expansion of hPSCs.

2. Materials and methods

2.1. Fabrication of a nanofibrous gelatin substrate

Gelatin solutions were prepared by dissolving gelatin in a mixture of acetic acid, ethyl acetate and distilled water (Table S1) 16 h prior to electrospinning. The solution was loaded into a syringe with a stainless steel 23-gauge needle. The distance between the needle and the collector (silicon wafer) was kept at 10 cm. A culture cover glass (ϕ 25 mm; Matsunami Glass Ind., Ltd.) was fixed in the center of the silicon wafer. A voltage of 11 kV was applied to the needle using a high-voltage power supply (TechDempaz). The gelatin solution was ejected with a syringe pump at a flow rate of 0.20 mL h^{-1}. All our electrospinning experiments were performed at 30 °C with a humidity level of 30%. After electrospinning, the samples were dried under vacuum at 37 °C overnight to evaporate the remaining solvent.

The electrospun gelatin nanofibers were cross-linked in 0.2 M EDC and 0.2 M NHS in ethanol for 4 h. After cross-linking, samples were rinsed with 99.5% ethanol 3 times and dried under vacuum at 37 °C overnight to remove the remaining cross-linking buffer.

2.2. Human pluripotent stem cell culture

hESCs were used following the Kyoto University guidelines. hESC (H9 and H1) and hiPSC (253G1) lines were maintained in Primate ES medium (ReproCELL) supplemented with 5 ng mL^{-1} human recombinant basic fibroblast growth factor (Wako Chemicals) and 100 U mL^{-1} penicillin/streptomycin. Inactivated mouse embryonic fibroblasts (1×10^4 cells cm^{-2}) were used to co-culture hPSCs. Media were changed every day, and hPSCs were passaged every 4–7 d using StemPRO EZ passage (Invitrogen).

2.3. Long-term expansion of hPSCs on nanofibrous gelatin substrates

hPSCs were harvested by treatment with TrypLE Express (GIBCO) for 5 min at 37 °C. Cells were then dissociated by pipetting (>10 times) and placed on the surface of nanofibrous gelatin substrates at 4×10^4 cells cm^{-2} in mTeSR-1 (STEM CELL Technologies), which is a defined serum-free medium containing 10 μM ROCK inhibitor (Y-27632; Wako Chemicals). The medium was changed to medium without Y-27632 after 48 h. Cells were then passaged once every 3–6 d by treatment with a non-enzymatic cell dissociation solution for 5 min at 37 °C and transferred onto a new nanofibrous gelatin substrate, which was sterilized with 99.5% ethanol three times before use. Before seeding cells, the substrate was coated with pre-warmed culture medium.

2.4. Alkaline phosphatase staining

Alkaline phosphatase (AP) staining was carried out using a Red-color AP staining kit (System Biosciences) following the manufacturer's instructions. Briefly, hPSCs were fixed with 4% v/v paraformaldehyde for 2 min at room temperature and then washed with phosphate-buffered saline (PBS) twice. Next, hPSCs were incubated with the AP substrate at room temperature for 30 min. All steps were performed in dark to protect the reagents from light exposure.

2.5. Immunocytochemistry

Cells were fixed in 4% v/v paraformaldehyde at room temperature for 30 min, permeabilized with 0.5% v/v Triton X-100 in D-PBS at 30 min at room temperature or 4 °C overnight, and incubated with blocking solution containing 5% v/v normal goat serum, 5% v/v normal donkey serum, 3% v/v bovine serum albumin, and 0.1% v/v Tween 20 in D-PBS at 4 °C overnight. Cells were then incubated with primary antibodies, i.e., anti-OCT4 (2 μg mL^{-1}), anti-NANOG (9.4 μg mL^{-1}), anti-SOX17 (20 μg mL^{-1}), anti-β-tubulin III (6 μg mL^{-1}), or anti-alpha smooth muscle actin (2 μg mL^{-1}) in 0.5 v/v % Triton X100 in D-PBS at 4 °C overnight. Following incubation with the primary antibody, cells were incubated with the appropriate secondary antibody, i.e., DyLight-649 anti-rabbit IgG (0.375 or 3 μg mL^{-1}) or DyLight 488 anti-mouse IgG (1.5 μg mL^{-1}), in blocking buffer at room temperature for 1 h. Finally, cell nuclei were stained with 300 nM 4′-6-diamidino-2-phenylindole (DAPI) at room temperature for 30 min.

2.6. Flow cytometry

hPSCs cultured on nanofibrous gelatin and Matrigel-coated substrates were harvested with TrypLE Express. Cells were washed twice with D-PBS and cell numbers were counted. For staining with an antibody, cells were diluted to a final concentration of 1×10^7 cells mL^{-1} in PBS containing 2% fetal calf serum. A phycoerythrin-labeled anti-human SSEA-4 antibody was added and cells were incubated at room temperature for 30 min. As a negative control, a specific isotype control was used. After removing the antibodies by centrifugation at 500 × *g* for 5 min, cells were washed with PBS containing 2% fetal calf serum. Finally, cell suspensions were applied to a FACS Canto II flow cytometer (BD Biosciences).

2.7. Total RNA purification

Total cellular RNAs were harvested using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. mRNA concentrations were measured in a NanoDrop1000 spectrophotometer (Thermo Fisher).

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

A mixture (25 μL) containing 1 μL cDNA, 0.2 μM PCR primers (Table S2) and 5 U of Taq DNA polymerase (Takara) in PCR buffer was subjected to amplification in a DNA thermal cycler (Applied Biosystems 7300 Real-Time PCR System). PCR was performed for 30–35 cycles (94 °C, 30 s; 58 °C, 30 s; 72 °C, 60 s) with an initial incubation at 94 °C for 5 min and a final extension at 68 °C for 3 min. Amplified products (10 μL) were resolved by 1.2% (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining. RT-PCR results of glyceraldehyde-3-phosphate dehydrogenase were used as control.

2.9. Quantitative RT-PCR

Total RNA was reverse transcribed using the RT First Strand Kit (Qiagen). cDNA was amplified and quantified with Power SYBR Green PCR MasterMix (Life Technologies) in combination with the Human Focal Adhesions PCR Array (Qiagen, PAHS-145Z) in a 96-well format (see also in Table S3) following the manufacturer's instructions. Briefly, cDNA solution was mixed with Power SYBR Green PCR MasterMix and applied to the PCR arrays. For amplification and quantification of cDNA, "hot start" PCRs were carried out with an initial incubation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and then 60 °C for 3 min using an Applied Biosystems 7300 Real-Time PCR system (Life Technologies). Data analysis was carried out using Cluster 3.0 in combination with the TreeView 1.1.5 open-source software.

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