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Culturing of mouse and human cells on soft substrates promote the expression of stem cell markers

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Substrate elasticity is a potent regulator of the cell state. Soft substrates have been shown to promote the homogeneous self-renewal of mouse embryonic stem cells through the down-regulation of cell-matrix tractions. We therefore investigated whether soft substrates promote the reprogramming of somatic cells into induced pluripotent stem (iPS) cells. After retroviral infection with five factors, Oct3/4, Klf4, Sox2, Lin28 and Nanog, mouse embryonic fibroblasts (MEFs) were cultured on several artificial substrates of varying elasticity and examined for the expression of pluripotency genes. When MEFs were cultured on soft (<0.1 kPa) polyacrylamide gels coated with gelatin, the expressions of Nanog and Oct3/4 genes were higher than in cells cultured on rigid plastic dishes (~10^6 kPa). The same result was obtained at higher elasticity (0.5 kPa) for adult human dermal fibroblasts (HDFa). We also examined whether reprogramming could be enhanced on soft substrates without exogenous gene introduction, finding that cells cultured on soft substrates in the presence of chemicals known to promote cell reprogramming exhibited up-regulated stem cell markers. These results suggest that controlling the substrate stiffness can enhance the initiation of cell reprogramming, which may lead to effective and reproducible iPS cell production.

[**Key words:** Induced pluripotent stem cells; Reprogramming; Elasticity; Fibroblasts; Actin filament]

Perhaps nothing has advanced the field of regenerative medicine more in recent years than the generation of induced pluripotent stem (iPS) cells (1). However, current iPS technology is still far from ideal. The efficiency of iPS generation remains very low despite techniques that suppress the p53 pathway (2) or induce hypoxia (3). Furthermore, the quality of iPS cells produced in different laboratories varies based on the levels of X chromosome inactivation (4,5). The genomic integration of transcription factors is also problematic, as this can lead to an increased risk of mutations and/or cancer. In addition, the iPS generation protocol is long, as it takes approximately 1 month to be completed (1). Although several methods have aimed to overcome these problems, including the exclusion of exogenous genes when the cell is reprogrammed (6,7), an efficient, fast, and reliable method for iPS generation is required.

Feeder cells are essential for establishing iPS cells. The demand for feeder cells implies that external factors, such as secreted chemokines and cell adhesion, are involved in the reprogramming of somatic cells. However, in addition to these biochemical factors, mechanical factors, such as the structure surrounding the cell, may also be important in cell reprogramming (8–10). Another mechanical factor is the substrate elasticity, which is also known to regulate the cell state. During stem cell differentiation, for example, mesenchymal stem cells (MSCs) determine their specific lineage with extreme sensitivity to the tissue-level elasticity (11). Moreover, a recent report showed that soft substrates promote the homogeneous self-renewal of mouse embryonic stem cells (mES cells) by reducing cell-matrix tractions (12). Although these reports demonstrate the importance of substrate stiffness in the fate of stem cells, the effect of substrate stiffness on cell reprogramming has not been reported.

To investigate whether substrate elasticity affects the initiation of cell reprogramming, we cultured cells on soft substrates after retroviral infection with the Yamanaka factors (13–15), finding that soft substrates promote the expression of stem cell markers. Soft substrates inhibited the expression of ROCK2, an effect that may have enhanced the survival of cells expressing the stem cell markers (16,17). Soft substrates also enhanced the expression of Oct3/4 and Nanog with the help of chemicals known to enhance cell reprogramming, but without any introduction of exogenous genes. The present study therefore demonstrates that soft substrates may improve the efficiency of generating and maintaining iPS cells.

MATERIALS AND METHODS

Cell culture Primary MEFs were purchased from Millipore (Millipore, Billerica, MA, USA) and cultured according to the supplier's recommendation. MEFs were maintained in high glucose DMEM (11960, Gibco, Gaithersburg, MD, USA) containing 10% FBS (16141, Gibco), 1% penicillin (Sigma–Aldrich, St. Louis, MO, USA), 1% streptomycin (Sigma–Aldrich), 1% GlutaMAX-1 (Gibco), 1% non-essential amino acid (Gibco), 1% nucleosides (Millipore), 1% sodium pyruvate (Sigma–Aldrich), 0.1% 2-mercaptoethanol (Sigma–Aldrich), and 0.1% leukemia inhibitory factor (LIF) (Sigma–Aldrich).

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Prior to seeding the cells, the dishes were coated with 0.1% gelatin solution (Millipore). Adult human dermal fibroblasts (HDFa) were purchased from ScienCell (ScienCell Research Laboratories, Carlsbad, CA, USA), cultured according to the supplier's recommendation, and grown until fifteen doublings of the cell number. Prior to seeding the cells, the dishes were coated with poly-L-lysine (2 μg/cm^2) (Sigma–Aldrich), and the cells were maintained in low glucose DMEM (D6046, Sigma–Aldrich) containing 10% FBS (16141, Gibco), 1% penicillin (Sigma–Aldrich), and 1% streptomycin (Sigma–Aldrich).

Induction of reprogramming The all-in-one lentivirus vector system (pDON-5 OKSLN) was used to induce reprogramming (Takara, Japan). In order to package the virus, G3T-hi cells were seeded at 2×10^6 cells in a 60-mm dish and co-transfected with pDon-5 OKSLN, pGP, and pE-ampho vectors using the FuGene6 transfection reagent (Promega) according to the manufacturer's instructions. After 48 h, the supernatant was collected and used to infect MEFs or HDFa seeded at 3×10^4 cells/35-mm dish. To enhance the viral infection, 4 μg/cm^2 of RetroNectin (Takara, Japan) was used. Four days after seeding, the infected cells were seeded at 5×10^4 cells/35-mm dish on artificial substrates with varying rigidity. LIF (0.1%) was added to the MEF culture media and 25 ng/mL fibroblast growth factor (bFGF) (Sigma–Aldrich) was added to the HDFa culture media. The induction of reprogramming by chemical compounds was performed as described previously (18). Cells were seeded at 5×10^4 cells/35-mm dish on artificial substrates of varying rigidity. Then, four chemical compounds were added to MEF culture media with 25 ng/mL bFGF (Sigma–Aldrich). The concentrations of the chemical inhibitors (noted as 4i throughout the paper) were 0.25 mM Sodium Butyrate (NaB) (Stemgent, Cambridge, MA, USA), 5 μM PS48 (Stemgent), 0.5 μM A-83-01 (Stemgent), and 0.5 μM PD0325901 (Stemgent). The medium including 4i was exchanged every other day.

Preparation of artificial substrates of varying rigidity Acrylamide substrate of varying rigidity was prepared according to a previously reported method (19) with slight modifications. Circular glass coverslips with diameters of 25-mm (Fisher Scientific, Germany) were washed with ethanol, dried, and coated with silane using 3-methacryloxypropyltriethoxysilane for 30 min. Glass coverslips were then washed again with ethanol and dried. The acrylamide/bisacrylamide mixture (8.5 μL) with TEMED and APS was dropped onto a glass coverslip and another coverslip with diameter of 22-mm was placed on top to sandwich the acrylamide/bisacrylamide mixture. The mixture was subsequently incubated for 1 h at room temperature. The rigidity of the substrate was controlled by varying the ratio of acrylamide and bisacrylamide (Table S1), and actual stiffness was measured using an atomic force microscope (NanoWizard 3, JPK Instruments, Berlin, Germany). After confirming that the acrylamide gel had solidified, the 22-mm glass coverslip was removed, leaving the acrylamide gel on the 25-mm glass coverslip. The acrylamide gel was washed twice with 50 mM HEPES buffer (pH 8.5). Then, 500 μL of 0.05% Sulfo-SANPAN solution was dropped onto the acrylamide gel, which was subsequently UV-irradiated (312 nm) for 10 min. The acrylamide gel was washed once with 50 mM HEPES buffer and soaked overnight in 50 mM HEPES buffer containing 0.1% gelatin. The height of the soft (<0.1 kPa), 0.5 kPa, and 4 kPa gels was 13.5 ± 3.8 μm, 11.8 ± 5.9 μm, 27.6 ± 13.8 μm, respectively.

Quantitative real-time RT-PCR Cells were sampled 4 days after seeding on an artificial substrate for RNA isolation (Fig. S1). RNA was isolated using an RNeasy mini kit (Qiagen, Germany), and cDNA was synthesized using an Omniscript RT Kit (Qiagen). cDNA was used for quantitative real-time polymerase chain reaction (PCR) analyses. The analyses were performed using primers corresponding to the *Mus musculus* and *Homo sapiens* sequences (Table S2). The real-time PCR mixture (8 μL) included 1× Quantities Eva Green Super Mix (Bio-Rad, Hercules, CA, USA), gene-specific primers (0.4 μM), and a cDNA template (300 ng) and was reacted in a CFX96 real-time PCR analysis system (Bio-Rad). The following PCR conditions were used: 98°C for 30 s, 40 cycles at 98°C for 5 s, 60.8°C (*m*Nanog and *h*Sox2), 59.6°C (*m*Sox2), 58.5°C (*m*RhoA, *m*Rock1 and *m*Rock2), 57.3°C (*m*Oct3/4 and *h*GAPDH), 55.0°C (*h*Nanog and *h*Oct3/4), 54.6°C (*h*ReX1) or 53.4°C (*m*GAPDH) for 5 s, 50°C for 5 s, and 95°C for 5 s.

Counting the number of colonies The samples were immuno-stained with Oct3/4 and Nanog antibodies and observed under a fluorescence microscope (BZ-9000, Keyence, Tokyo, Japan). More than five micrographs of dimension 724 μm × 546 μm were obtained from each sample. The colony number was visually counted from the micrographs according to the method of a previous publication (20). The Nanog and Oct3/4 positive colonies were determined by comparing the fluorescence intensity against a negative-control in which the samples were stained only with a secondary antibody.

Immunohistochemistry Cultured cells were washed once with PBS and then fixed with 4% paraformaldehyde (PFA) for 20 min at 4°C. Samples were washed for 5 min in PBS three times and then treated with CAS-BLOCK (Invitrogen, Carlsbad, CA, USA) solution for 30 min at room temperature. Primary antibodies were diluted with CAS-BLOCK and then incubated with the samples overnight at 4°C. The following antibodies were used at a dilution of 1:100; Nanog antibody (ab80892, Abcam, Cambridge, UK), Oct3/4 antibody (sc-5279, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Integrin α1 antibody (ab106267, Abcam, Cambridge, UK), Integrin α5 antibody (ab55988, Abcam, Cambridge, UK), and Integrin α7 antibody (sc-27710, Santa Cruz Biotechnology, Inc.). After rinsing for 5 min in PBS four times, samples were incubated at room temperature for 1.5 h with fluorescent secondary antibodies (Alexa-488 or Alexa-594, Invitrogen). Samples were then observed under a fluorescence microscope after rinsing for 5 min in PBS three times.

RESULTS AND DISCUSSION

Soft substrates promote the up-regulation of Nanog and Oct3/4 in MEFs To examine whether soft substrates promote the initiation of cell reprogramming of MEFs, we prepared three artificial substrates of varying elasticity (<0.1 kPa, 0.5 kPa, and 4 kPa) and compared the cells cultured on those substrates to those cultured on rigid surfaces (regular culture dish) and to mitomycin-C treated feeder cells. Cells were infected with the reprogramming factors Oct3/4, Klf4, Sox2, Lin28, and Nanog (OKSLN) and then seeded on the substrates. The cells showed different morphologies depending on the elasticity of the substrate (Fig. 1), which may be due to changes in the traction force generated by the cells. Cells on softer substrates (<0.1 kPa and

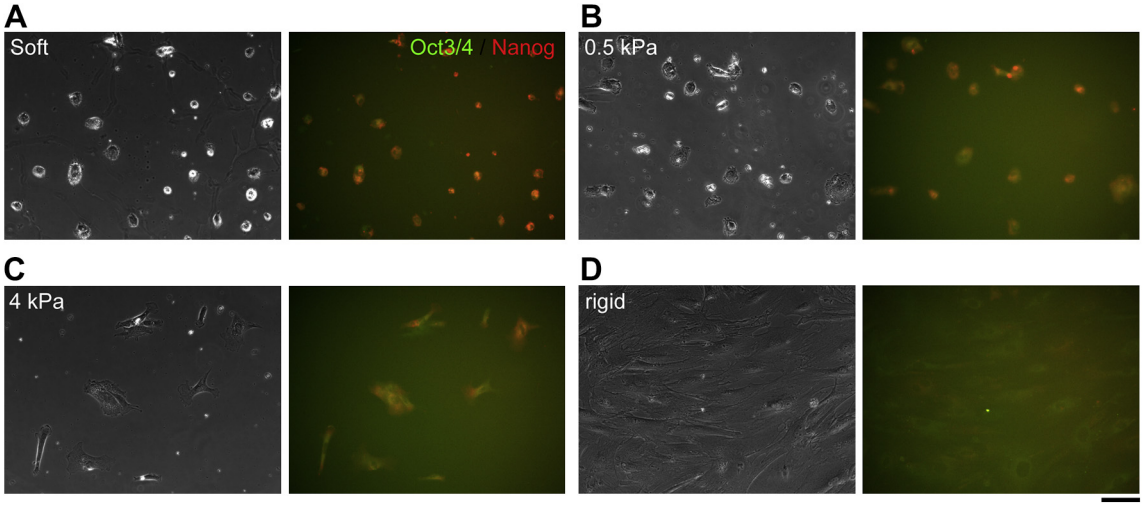

FIG. 1. Mouse fibroblasts cultured on soft substrates after infection with reprogramming factors. Phase contrast (left) and fluorescence (right) images of cells cultured on (A) <0.1 kPa (soft), (B) 0.5 kPa, (C) 4 kPa, and (D) rigid substrates 4 days after seeding. Cells were infected with reprogramming factors 4 days prior to seeding and subsequently stained with Oct3/4 (green) and Nanog (red) antibodies. Scale bar: 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)