Supplementary Information

Dynamic Control of Cell Adhesion on a Stiffness-Tunable Substrate for Analyzing the Mechanobiology of Collective Cell Migration

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Materials and methods

1. Materials

Photocleavable poly(ethylene glycol) (PCP) was synthesized as described in our previous report.[1,2] Methanol, ethanol, toluene, acrylamide, *N,N′*-methylenebis(acrylamide), *N,N,N′,N′*-tetramethylethylenediamine, and penicillin-streptomycin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Ammonium peroxodisulfate, 3-(trimethoxysilyl)propyl methacrylate, and n-octadecyltrichlorosilane were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate (sulfo-SANPAH) was purchased from Thermo Fisher Scientific Co. (Waltham, MA, USA). Poly-D-lysine (PDL; M_w= 30,000-70,000 g mol^{-1}), fluorescein isothiocyanate (FITC), FITC-labeled bovine serum albumin (FITC-BSA), minimum essential medium (MEM), and L-glutamine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum was purchased from BioWest (Nuaille, France). FluoSpheres Carboxylate-Modified Microspheres (fluorescent beads, diameter = 0.02 μm, yellow-green fluorescent (505/515)), MEM non-essential amino acids, and trypsin-EDTA solution were purchased from Invitrogen (Carlsbad, CA, USA). Human fibronectin was purchased from Corning (Corning, NY, USA). Glass coverslips were purchased from Matsunami (18×18 mm^2, 0.12–0.17 mm thick, Osaka, Japan). SUS304 steel balls (diameter = 0.5 mm) were purchased from Funabe Seiko (Nishinomiya, Japan). All chemicals were used as supplied, without further purification.

2. Preparation of chemically modified glass coverslips

Surface-functionalized glass coverslips with various chemical modifications were prepared as follows. First, the surface of the coverslips was cleaned with a UV-O_3 cleaner for 30 min. Methacrylate-modified glass coverslips were prepared as follows: cleaned glass coverslips were immersed in a 0.4 wt% aqueous solution of

3-methacryloxypropyltrimethoxysilane for 1 h, washed with ultrapure water three times, and dried in air at room temperature. Hydrophobic glass coverslips were prepared as follows: cleaned glass coverslips were immersed in a 1 wt% n-octadecyltrichlorosilane toluene solution for 1 h, washed with methanol three times, and dried in air at room temperature overnight.

3. Preparation and functionalization of stiffness-controlled photoactivatable substrates

Stiffness-controlled photoactivatable substrates were prepared by polymerizing polyacrylamide solutions between the acrylated and hydrophobized glass coverslips, according to a slightly modified version of a previously reported procedure.[3] Specifically, a methacrylate-modified glass coverslip was cut into pieces (ca. 100 mm^2). A solution containing 0.12% ammonium peroxodisulfate, 0.15% *N,N,N′,N′*-tetramethylethylenediamine, and acrylamide and *N,N′*-methylenebis(acrylamide) at the appropriate ratio (Table S1) was prepared. A 10 μL droplet of the polyacrylamide solution, which remained fluid for at least 30 s of mixing, was immediately pipetted onto a hydrophobic glass coverslip. The methacrylate-modified glass coverslip was then carefully placed on top of the droplet. The polymerization was completed in about 5 min, at which point the hydrophobic glass coverslip was slowly peeled off, and then the gel surface was washed three times with ultrapure water and three times with HEPES buffer (50 mM, pH 8.5). Then 40 μL of sulfo-SANPAH solution (1 mg mL^{-1}, 50 mM HEPES, pH 8.5) was pipetted onto the prepared gel surface and activated by means of UV (365 nm) irradiation for 5 min. The excess sulfo-SANPAH solution was removed by washing three times with ultrapure water. Then the gel substrate was immersed in an aqueous solution of PDL (1 mg mL^{-1}) and incubated overnight at 50°C. The PDL-modified substrate was then washed three times with ultrapure water. The surface was soaked in an ultrapure water solution containing PCP5k (1 mg mL^{-1}) and allowed to react overnight at 50°C. Unreacted polymer was removed from the surface by washing three times with ultrapure water. Then the surface was treated with a PCP2k solution in ultrapure water (1 mg mL^{-1}) in a similar fashion. The obtained PCP5k/2k-PDL-modified gel surface was stored in ultrapure water until use.

The stiffness of the bare-gel substrate and the PDL-modified gel substrate was characterized by means of a gel indentation assay, as described previously.[4] Briefly, a commercially supplied fluorescent bead solution was diluted with ultrapure water at a 1:10,000 ratio; the diluted solution was pipetted onto the surface of the gel substrate to infuse into the gel for 1 min at room temperature; and then the surface was washed three times with ultrapure water. Then a steel ball was gently placed on the surface of the gel substrate by means of a pipette, and the focal plane of an IX81-PAFM fluorescence microscope (Olympus, Tokyo, Japan) was adjusted so that a clear image of some of the fluorescent beads located under the steel ball was visible by confocal fluorescence observation using a disk scan unit (CSU-10, Yokokawa, Ishikawa, Japan) and a cooled CCD camera (Rolera MGi, Q-Imaging, Burnaby, BC, Canada). The z-position was recorded using the MetaMorph image-processing system (Molecular Devices, Downingtown, PA, USA). After the steel ball was blown off with water from a pipette, the plane of focus was again adjusted to the same fluorescent beads, and the z-position was measured. The elastic modulus of the PDL-modified gel substrates was calculated from the average differences between the two z-positions, according to the previously reported procedure.[4]

Table S1. Ratio of acrylamide/bisacrylamide

Sample (Expected Young's modulus)	Acrylamide (%)	Bisacrylamide (%)
Soft gel (5 kPa)	7.5	0.2
Stiff gel (55 kPa)	12	0.6

4. Photoirradiation of the substrates for cell patterning

PEGylated surfaces were irradiated under the IX81-PAFM fluorescence microscope equipped with a UPLSAPO 10× objective lens (Olympus) and an excitation filter (377 ± 25 nm; Omega, Brattleboro, VT, USA). The power density of the UV light was measured with a UIT-150 power meter equipped with a UVD-S365 sensor (Ushio, Tokyo, Japan). The irradiation dose was kept at 10 J cm^{-2}; at this dose the photocleavage reaction of the 2-nitrobenzyl ester is almost completed based on our previous study.[1] The photoirradiation pattern was controlled by inserting a photomask printed on a transparency at the position of the field diaphragm.[5,6] The functionalized photoactivatable substrates were placed in a 35 mm glass-bottom dish (MatTek, MA, USA) and soaked in PBS for the irradiation. The photoirradiation procedure used to induce cell migration is described below.

5. Measurement of ζ-potentials

The surface charge of the substrates was measured with a ζ-potential analyzer (Delsa Nano C, Beckman Coulter, CA, USA) equipped with a flat surface cell. The monitoring polystyrene particles were suspended in 10 mM NaCl (pH 7.0), and the mobility of the particles under an electric field was detected along the vertical direction toward the center of the sample cell by using the Doppler shift of the laser.

6. Quantitative analysis of protein adsorption

Protein adsorption on the surface of the substrates was evaluated using FITC-BSA and FITC-labeled fibronectin (FITC-fibronectin) as model proteins. FITC-fibronectin was synthesized as described previously, with slight modifications.[7,8] Briefly, fibronectin (1 mg, 4×10^{-9} mol) and FITC (0.01 mg, 20×10^{-9} mol) were stirred in 2 mL of carbonate buffer (50 mM, pH 9.0) overnight at room temperature. The obtained FITC-fibronectin was purified on a PD-10 column (GE Healthcare, Little Chalfont, UK). FITC-BSA or FITC-fibronectin (1 mg mL^{-1} in PBS) was deposited on the surface of the substrates, which were then held at room temperature for 3 h. After the substrates were washed five times with PBS, fluorescence images were captured with a cooled CCD camera (Retiga-Exi, Q-Imaging) under a fluorescence microscope using the following set of barrier filters (Omega): 485DF15, 505DRLPXR, and 510AF23. The obtained fluorescence images were analyzed by means of the MetaMorph image-processing system, and the amount of adsorbed FITC-BSA was determined from the fluorescence intensity after background subtraction.

7. Cell culture and patterning on the photoactivatable substrates

Madin–Darby canine kidney (MDCK) epithelial cells (RCB0995, RIKEN Cell bank) were cultured at 37°C under 5% CO_2 in MEM supplemented with 10% fetal bovine serum, 1% MEM non-essential amino acids, 1% penicillin-streptomycin, and L-glutamine. Prior to passaging and prior to the experiments, cells were detached with a trypsin-EDTA solution. For cell patterning, cells were seeded on the photoirradiated surfaces at 1×10^6 cells/dish in 35 mm glass-bottom dishes. These seeding densities correspond to 1×10^5 cells cm^{-2}. Unattached cells on the substrates were removed after 2 h by replacing the medium. The cells were cultured for 8 h to reach confluence within the spot, and then cell migration was induced by irradiating some or all of the surrounding regions (but not over the patterned cells)[5,6,9] at 10 J cm^{-2}. Cell migration was periodically imaged with the above-mentioned microscope, and the images were analyzed with the MetaMorph software.

8. Particle image velocimetry-like analysis

From phase-contrast images, particle image velocimetry-like analysis was performed with a MATLAB program written in our laboratory. Instead of cellular velocity, we analyzed cellular displacement vector over the image field by template matching[10]. We estimated the cellular displacement field based on the normalized cross-correlation (NCC) function for each 10 pixel in the image I with size $M \times N$ at time $t + dt$ according to the template image T with size $U \times V$ at time t:

$$NCC(x,y) = \frac{\sum_{u,v}\left(I(x+u,y+v)-\bar{I}\right)\left(T(u,v)-\bar{T}\right)}{\sqrt{\sum_{u,v}\sum_{u,v}\left(I(x+u,y+v)-\bar{I}\right)^2 \times \left(T(u,v)-\bar{T}\right)^2}}$$

where, $x = 0, 1, 2, \cdots, M-1$, $y = 0, 1, 2, \cdots, N-1$, $u = 0, 1, 2, \cdots, U-1$, and $v = 0, 1, 2, \cdots, V-1$. $\bar{T}$ represents the mean of T and $\bar{I}$ is the mean of I. We set dt to be 5 min (= 1 frame) with 25×25 pixels for the template size (1.27 μm/pixel).

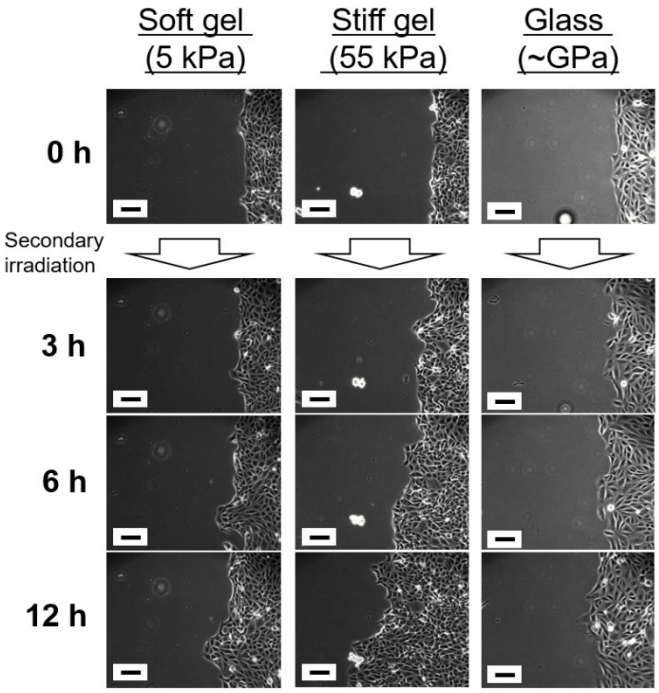

Figure S1. Stiffness- and time-dependent cell migration behavior of Madin–Darby canine kidney epithelial cells before and after induction of geometrically constrained cell migration. The cells were patterned in rectangular cell-adhesive regions, and their migration was induced by the secondary irradiation in the region on the left side of the cells 10 h after cell seeding (0 h, before release). Scale bar: 100 μm.

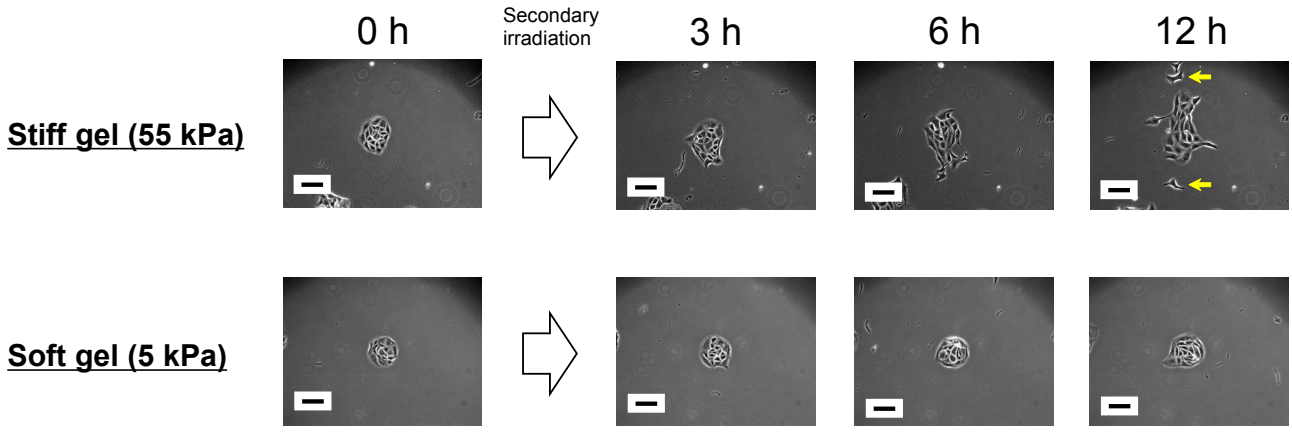

Figure S2. Impact of gel stiffness on cluster expansion behaviors from low cell density clusters. Cells were initially confined in 100-μm circular spots by the first irradiation and their migration was induced by secondary irradiation of their surroundings. Arrowheads indicate cells dissociated from the cluster. Cell density, 20-30 cells/cluster. Scale bars, 100 μm.

Movie S1. Dynamic patterning of MDCK cells on the photoactivatable stiff-gel substrate. The cells were initially patterned in a rectangular regions, and their migration was induced by the secondary irradiation of the

region on the left side of the cells 10 h after cell seeding (0 h, before release). Scale bars, 100 μm.

Movie S2. Time-course of migration from a confluent rectangular cluster of MDCK cells on the photoactivatable stiff-gel substrate. Scale bars, 100 μm.

Movie S3. Time-course of migration from a confluent rectangular cluster of MDCK cells on the photoactivatable soft-gel substrate. Scale bars, 100 μm.

Movie S4. Time-course of migration from a circular cluster of MDCK cells on the photoactivatable stiff-gel substrate. Scale bars, 100 μm.

Movie S5. Time-course of migration from a circular cluster of MDCK cells on the photoactivatable soft-gel substrate. Scale bars, 100 μm.

Movie S6. Particle image velocimetry–like representation of the cellular flow shown in Movie S4.

Movie S7. Typical migration behaviors of single MDCK cells sparsely seeded on the stiff-gel substrate. Scale bars, 100 μm.

Movie S8. Typical migration behaviors of single MDCK cells sparsely seeded on the soft-gel substrate. Scale bars, 100 μm.

Movie S9. Time-course of migration from a circular cluster of MDCK cells with low cell density on the photoactivatable stiff-gel substrate. Scale bars, 100 μm.

Movie S10. Time-course of migration from a circular cluster of MDCK cells with low cell density on the photoactivatable soft-gel substrate. Scale bars, 100 μm.

All movies are shown at 3000× speed.

References

(1) S. Kaneko, H. Nakayama, Y. Yoshino, D. Fushimi, K. Yamaguchi, Y. Horiike, J. Nakanishi, *Phys. Chem. Chem. Phys.*, 2011, **13**, 4051.

(2) M. Kamimura, O. Scheideler, S. Shimizu, S. Yamamoto, K. Yamaguchi, J. Nakanishi, *Phys. Chem. Chem. Phys.*, 2015, **17**, 14159.

(3) R. S. Fischer, K. A. Myers, M. L. Gardel, C. M. Waterman, *Nat. Protoc.*, 2012, **7**, 2056.

(4) R. Long, M. S. Hall, M. Wu, C. Hui, *Biophys. J.*, 2011, **101**, 643.

(5) J. Nakanishi, Y. Kikuchi, T. Takarada, H. Nakayama, K. Yamaguchi, M. Maeda, *J. Am. Chem. Soc.*, 2004, **126**, 16314.
(6) J. Nakanishi, *Micropatterning in Cell Biology, Methods in Cell Biology Part B*, 2014, **120**, 117.
(7) M. Pallis, R. A. Robins, R. J. Powell, *Cytometry*, 1997, **28**, 157.
(8) C. Hoffmann, J. Leroy-Dudal, S. Patel, O. Gallet, E. Pauthe, *Anal. Biochem.*, 2008, **372**, 62.
(9) C. G. Rolli, H. Nakayama, K. Yamaguchi, J. P. Spatz, R. Kemkemer, J. Nakanishi, *Biomaterials*, 2012, **33**, 2409.
(10) J. P. Lewis, *Vision Interface* 1995, **10**, 120.