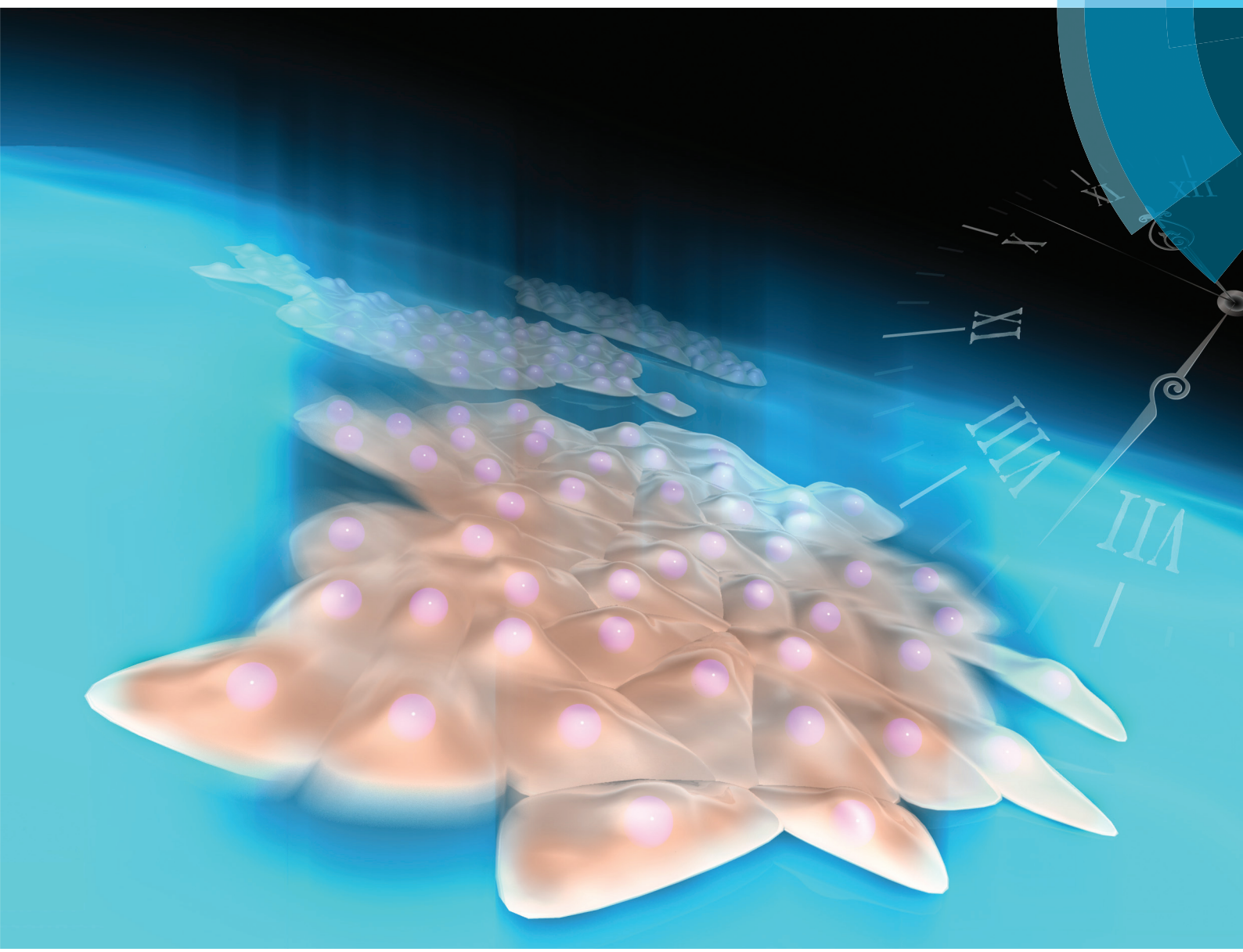
Volume 4 | Number 6 | June 2016 | Pages 883–1016

Biomaterials Science

www.rsc.org/biomaterialsscience



ISSN 2047-4830

ROYAL SOCIETY OF CHEMISTRY

COMMUNICATION

Jun Nakanishi *et al.*

Dynamic control of cell adhesion on a stiffness-tunable substrate for analyzing the mechanobiology of collective cell migration

iCeMS KYOTO UNIVERSITY WPI Research Center

Biomaterials Science

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Biomater. Sci.*, 2016, **4**, 933

Received 9th February 2016,
Accepted 29th March 2016

DOI: 10.1039/c6bm00100a

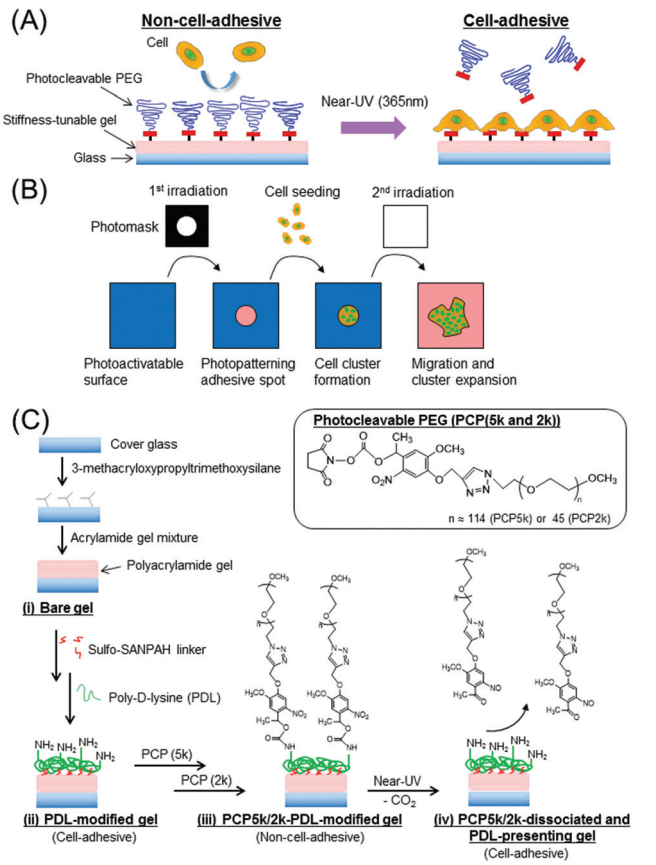
www.rsc.org/biomaterialsscience

Dynamic control of cell adhesion on a stiffness-tunable substrate for analyzing the mechanobiology of collective cell migration†

Masao Kamimura,[a] Michiko Sugawara,[b] Shota Yamamoto,[c] Kazuo Yamaguchi[c] and Jun Nakanishi*[a]

A method was developed for photocontrolling cell adhesion on a gel substrate with defined mechanical properties. Precise patterning of geometrically controlled cell clusters and their migration induction became possible by spatiotemporally controlled photoirradiation of the substrate. The clusters exhibited unique collective motion that depended on substrate stiffness and cluster geometry.

Collective migration is an important cellular activity involved in morphogenesis, wound healing, and cancer metastasis.[1,2] In a similar fashion to single-cell migration,[3] collective migration is affected not only by biochemical cues, but also by the mechanical properties of extracellular matrices (ECM).[4] Moreover, in the case of collective cell migration, tensile and compressive forces between cells and across multiple rows of cells play an important role,[5–7] emerging unique collective migration characteristics. For example, leader cell appearance,[8,9] vertical cellular movements,[10] left–right asymmetry,[11] and geometry-dependent drug responses[12] cannot be predicted simply by summing single-cell behaviors. Furthermore, recent studies identified some important proteins that coordinate collective cell migration through mechanotransduction pathways.[13–15] Some of these features have been already demonstrated to depend strongly on cell cluster geometry. Therefore, it is important to develop platforms for studying collective cell migration under conditions in which the material stiffness is defined and cellular geometries are well-controlled. To fulfill these requirements, we herein developed stiffness-tunable gel substrates whose surface was functionalized with photocleavable poly(ethylene glycol) (PEG) (Scheme 1A). In a similar fashion to our previous studies,[9,12,16–18] the gel surface changes from non-cell-adhesive to cell-adhesive due to the release of cell-repellent PEG from



Scheme 1 Schematic illustrations of the design of a photoactivatable gel substrate with defined mechanical properties. (A) Change in surface from non-cell-adhesive to cell-adhesive in response to near-UV irradiation. (B) Procedure for cell patterning and migration induction. Blue and pink represent non-cell-adhesive and cell-adhesive surfaces, respectively. (C) Preparation and photochemical reaction of the substrate.

[a]*WPI Research Center Initiative, International Center for Materials Nanoarchitectonics (MANA), National Institute for Materials Science (NIMS), 1-1 Namiki, Tsukuba 305-0044, Japan. E-mail: NAKANISHI.Jun@nims.go.jp*
[b]*Department of Mechanical Engineering, Graduate School of Engineering, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan*
[c]*Department of Chemistry, Faculty of Science, Research Institute for Photofunctionalized Materials, Kanagawa University, 2946 Tsuchiya, Hiratsuka, Kanagawa 259-1293, Japan*

† Electronic supplementary information (ESI) available: Full experimental details and additional characterization data. See DOI: 10.1039/c6bm00100a

the surface *via* the photocleavage of the 2-nitrobenzyl group. We are able to attach cells in arbitrary geometries corresponding to photoirradiation pattern and to induce cellular migration by the secondary irradiation of the initially idle regions (Scheme 1B).[9,12,16–19] The big difference from the previous substrates is that mechanical properties of the present substrate can be tuned close to that of soft tissues (0.1–100 kPa). We investigated the impact of substrate stiffness and cluster geometry on the collective migration behavior of Madin–Darby canine kidney (MDCK) epithelial cells.

The material design strategy is depicted in Scheme 1C. As a base material, we used a polyacrylamide gel, the Young's modulus of which can be tuned to match that of various soft tissues.[4,20] Then poly-D-lysine (PDL) was conjugated to the top surface of the gel *via* a sulfo-SANPAH linker to make the surface cell-adhesive (Scheme 1C(ii)).[20] Finally, photocleavable PEG (PCP) was conjugated to the amino groups of PDL to prepare a non-cell-adhesive surface. This last step made the stiffness-tunable substrate photoswitchable (Scheme 1C(iii) and (iv)). As a proof of concept, two gels with different stiffnesses (5 and 55 kPa) were prepared by choosing appropriate mixing ratios of acrylamide and *N*,*N*′-methylenebis(acrylamide) (Table S1†) based on the literature.[20] A gel indentation assay[21] indicated that the stiffnesses of the bare gels were 6.5 ± 1.5 and 53.0 ± 5.1 kPa, and these values agree well with our intended stiffnesses (Table 1). The mechanical properties did not change markedly following conjugation of the gel surfaces with PDL (Table 1; 5.7 ± 1.4 and 51.0 ± 6.4 kPa). Hereafter, we refer to these two gels as soft gels and stiff gels, respectively.

The functionalized polyacrylamide gel surfaces were characterized by means of ξ potential measurements. The bare gels showed almost neutral ξ potentials, and the PDL-functionalized gels showed positive ξ potentials (Fig. 1A(i) and (ii)). Upon addition of PCP5k alone or PCP5k followed by PCP2k, the ξ potentials of the gels dropped to almost zero (Fig. 1A(iii) and (iv)). These results indicate successful PDL modification and subsequent PEG grafting onto the PDL-modified surface to shield the surface charge in a fashion similar to that reported previously.[12] Additionally, sequential PEGylation with long and short PEG chains is known to improve the protein repellency of the surface.[12,22]

To evaluate the photoswitchability of the surface, we measured the change in adsorption of fluorescently-labeled fibronectin. The adsorption profiles of the soft and stiff gels were similar (Fig. 1B). The negatively charged fibronectin adsorbed strongly to the positively charged PDL-modified gels (Fig. 1B(ii)); and adsorption was blocked by PEGylation

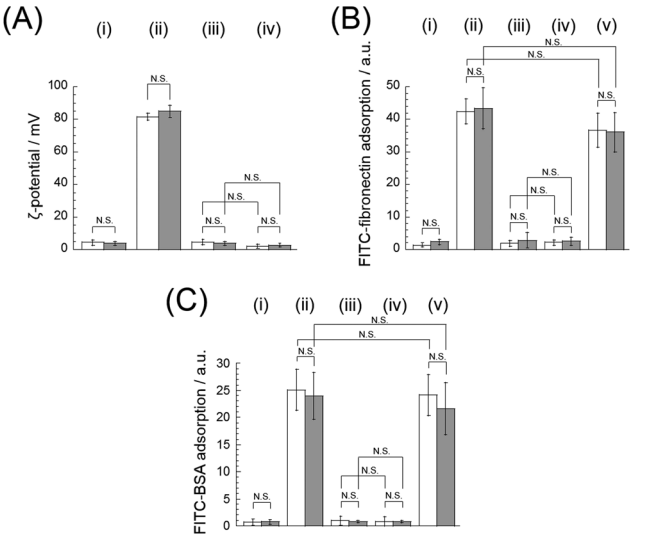


Fig. 1 Surface characterization of photoactivatable gel substrates. (A) ξ potentials, (B) FITC-fibronectin adsorption, and (C) FITC-BSA adsorption results for (i) bare gel, (ii) PDL-modified gel, (iii) PCP5k-PDL-modified gel, (iv) PCP5k/2k-PDL-modified gel, and (v) PCP5k/2k-PDL-modified glass after photoirradiation at 10 J cm^{-2}. White bars, soft gel; gray bars, stiff gel. Data are means ± SDs (n = 3). NS indicates nonsignificance; statistical significance was evaluated using Student's t-test.

(Fig. 1B(iii) and (iv)) but returned to a level comparable to that observed for the original PDL-modified surface after near-UV irradiation (Fig. 1B(v)). Similar results were obtained for bovine serum albumin (Fig. 1C). These results led us to conclude that PCP5k and PCP2k did in fact passivate the PDL-modified gels and make the gel surfaces photoresponsive. Note that the ξ potential analysis and protein adsorption studies showed no statistically significant difference between the soft and stiff gels at any of the functionalization steps or after photocleavage (Fig. 1A–C). That is, our surface functionalization procedures provided polyacrylamide gels with different mechanical properties but identical surface chemistries.

The prepared gel substrates were then used for cell-adhesion studies. Clusters of MDCK cells were confined in various geometrical patterns corresponding to the irradiation patterns (Fig. 2A). To demonstrate that gels could be dynamically patterned, we selectively irradiated the open space to the left of the rectangular cluster to induce collective cell migration (Fig. 2B and Movie S1†). The cluster expanded only from the left-hand boundary; the right-hand boundary of the cluster was unchanged. Although the cells themselves were not irradiated, it should be emphasized that this dose of near-UV irradiation has little cytotoxicity, as reported in our previous paper.[17] Taken together, these results demonstrate that our photoactivatable gel substrates could be used not only to pattern cells in arbitrary geometries (static patterning) but also to induce cell migration by activating regions adjacent to the patterned cells by means of secondary photoirradiation (dynamic patterning).

Table 1 Gel indentation assay results for bare gel and PDL-modified gel

		(Soft gel)	(Stiff gel)
Expected stiffness		5 kPa	55 kPa
Real stiffness	(Bare gel)	6.5 ± 1.5 kPa	53.0 ± 5.1 kPa
	(PDL-modified gel)	5.7 ± 1.4 kPa	51.0 ± 6.4 kPa

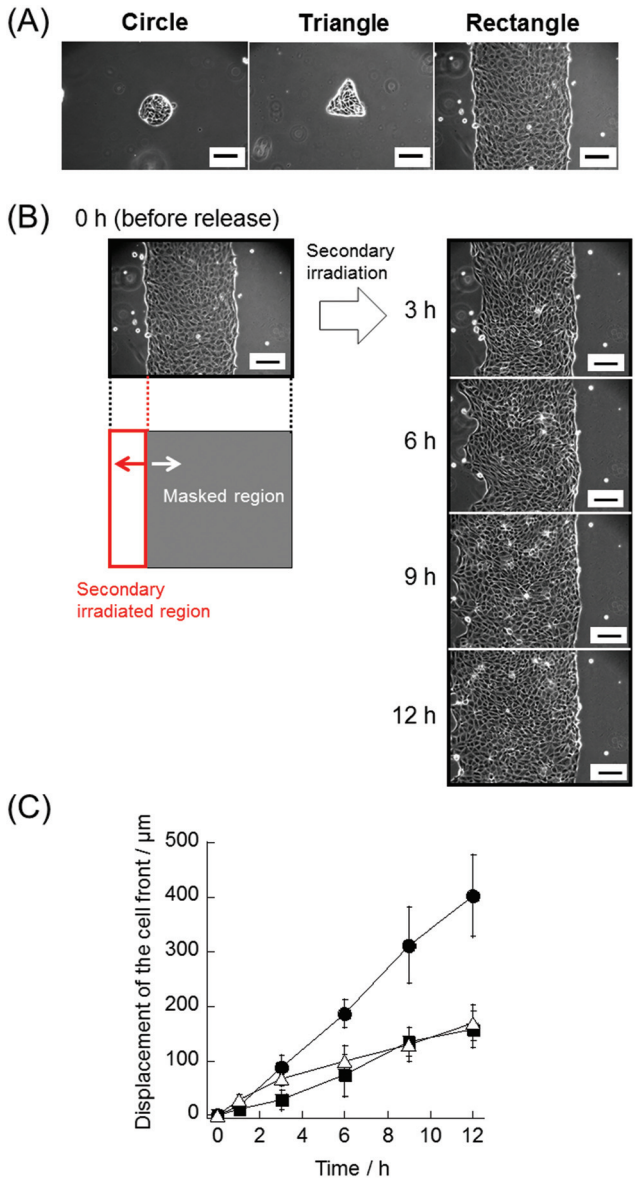


Fig. 2 Static and dynamic cell patterning on the photoactivatable gel substrates. (A) MDCK cells patterned in various geometries on the stiff gel. (B) An example of dynamic cell patterning on the stiff gel. The open space to the left of a rectangular cluster (0 h, before release) was selectively irradiated to induce migration (right). The time after the secondary irradiation is indicated. Scale bars, 100 μm. (C) Leading edge advancement of the rectangular clusters on stiff gel (filled circles), soft gel (filled squares), and glass (open triangles, control). Cell migration was induced in the same way as (B). Data are means ± SDs (n = 3).

We next examined the impact of substrate stiffness on collective cell migration. Cell migration on the soft- and stiff-gel substrates was induced in the manner described above. We chose this migration mode because our method can be compared with well-established conventional scratch wound healing assay. Plots of the average displacement of the cell cluster front *versus* time indicated that collective migration was by far faster on the stiff-gel substrate than on the soft-gel substrate (Fig. 2C, S1 and Movies S2, S3†). Given that the two gels have similar surface chemical properties and protein adsorption capabilities, the observed difference in migration rate reflects the dependence of collective cell migration on the mechanical properties of the substrate. Moreover, we also prepared a photoactivatable glass substrate, which has been developed in our previous report[12] and cell migration thereon was investigated (Fig. 2(C) and S1†). The surface design of this glass substrate is almost the same as those of the gel substrates reported in the current study; physically adsorbed PDL was functionalized with PCP5k/2k. The substrate possesses the same photoactivatable feature as the gel substrates, but its Young's modulus is extremely stiff (~GPa) compared to the gel substrates (~GPa). Cell migration rate on the glass substrate was again decelerated on the extremely stiff glass substrate to the level similar to the soft gel, indicating that the cells exhibit the fastest cell migration rate at the intermediate substrate stiffness. Similar results have been reported previously both for single-cell migration and for collective migration.[3,4,23,24] Our results indicate that our photoresponsive substrates are useful for investigating the effects of substrate stiffness on collective cell migration under defined cluster geometries.

Finally, we investigated the impact of substrate stiffness on the collective migration of cell clusters patterned in a defined circle of 100 μm in diameter. We previously demonstrated that the appearance of the leader cell depends strongly on the initial size of the cluster, as well as the curvature of its boundary. Therefore, studying the collective migration of cells with controlled cluster geometries is critical.[9] We focused on clusters with only 50–60 cells before migration induction. Cell migration from the circular clusters was induced by irradiation over the open regions surrounding the clusters (but not over the patterned cells) (Fig. 3). On the stiff gel, the cells exhibited wavy motions, like tidal ebb and flow, around the initial cluster area (Fig. 3A(ii) and Movie S4†). The particle image velocimetry (PIV)-like analysis further demonstrated that the cells on the stiff gel migrated aggressively and collectively in various directions with the occasional formation of complex vortices (Fig. 3B below and Movie S6†).

In contrast, cells on the soft-gel substrate exhibited distinct collective behavior (Fig. 3A(i) and Movie S5†). Although the regions surrounding the cells became cell-adhesive upon secondary near-UV irradiation, the cells were unable to expand from the initial circular region for approximately the first 6 h; the cells remained and proliferated within that region. After this priming period, the cells started to migrate out from the initial region, but only in one direction in an unusual avalanche-like motion (Fig. 3A(i)). Furthermore, cell migration from low cell density clusters (20–30 cells per cluster) showed different migration phenotypes (Fig. S2 and Movies S9, S10†). The cells did not migrate out from the initial circular spot within the observation time (12 h) on the soft gel, whereas some cells lost their connections with their original clusters and became isolated as single cells on the stiff gel (Fig. S2,† arrowheads). These results indicate not only gel stiffness, but also the initial cluster density determines migration pheno-

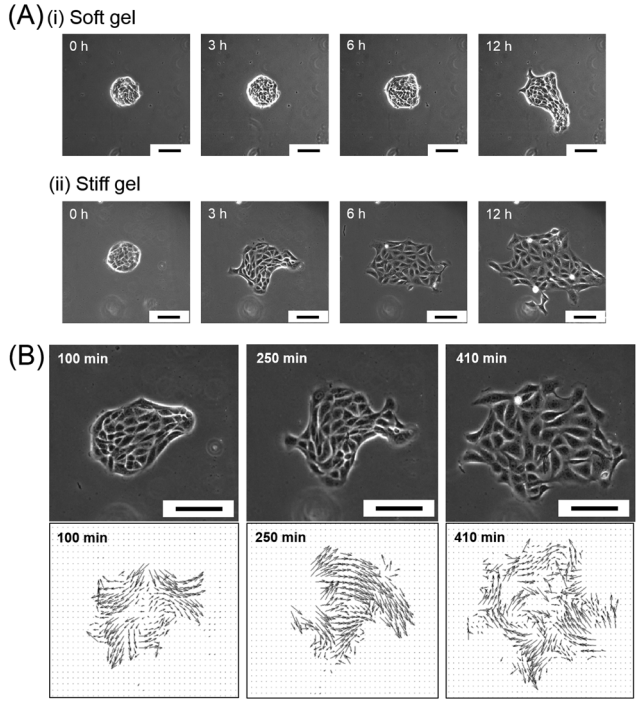


Fig. 3 Impact of gel stiffness on cluster expansion behaviors. (A) Cell migration behaviors on (i) soft gel and (ii) stiff gels. Cells were initially confined in 100 μm circular spots by the first irradiation and their migration was induced by secondary irradiation of their surroundings. (B) Particle image velocity-like analysis of the cell migration behavior on the stiff gel. Scale bars, 100 μm.

types. Further detailed analysis is now underway and it will be published in a forthcoming paper.

Note that isolated single cells seeded on the stiff and soft gels showed almost indistinguishable actively migrating behaviors (Movies S7 and S8†). Therefore, the observed difference in collective migration behavior on the soft- and stiff-gel substrates was due to mechanical sensitivity that emerged when the cells became a group. Further systematic studies with controlled cluster geometry and material stiffness will help to elucidate the mechanical regulation of collective migration. Moreover, the substrates used in the present study are compatible with traction force microscopy when fluorescent particles are embedded in polyacrylamide gels. These studies are now underway, and the results will be reported in forthcoming papers.

In summary, we used a novel stiffness-tunable gel substrate with a photoactivatable surface to demonstrate quantitative and qualitative differences in the collective migration behavior of cell clusters depending on their geometry as well as on the substrate stiffness. We expect this platform to be a promising and robust one for investigating the mechanobiology of collective cell migration.

Acknowledgements

This work was partially supported by the World Premier International Research Center (WPI) Initiative on Materials Nanoarchitectonics and by JSPS KAKENHI (no. 23750093, 25882048, and 15H03831). We thank Mr T. Komoda for helping with the PIV analysis.

Notes and references

1 P. Friedl and D. Gilmour, *Nat. Rev. Mol. Cell Biol.*, 2009, **10**, 445.
2 P. Rørth, *Annu. Rev. Cell Dev. Biol.*, 2009, **25**, 407.
3 S. R. Peyton and A. J. Putnam, *J. Cell. Physiol.*, 2005, **204**, 198.
4 M. R. Ng, A. Besser, G. Danuser and J. S. Brugge, *J. Cell Biol.*, 2012, **199**, 545.
5 X. Trepat, M. R. Wasserman, T. E. Angelini, E. Millet, D. A. Weitz, J. P. Butler and J. J. Fredberg, *Nat. Phys.*, 2009, **5**, 426.
6 D. T. Tambe, C. Corey Hardin, T. E. Angelini, K. Rajendran, C. Y. Park, X. Serra-Picamal, E. H. Zhou, M. H. Zaman, J. P. Butler, D. A. Weitz, J. J. Fredberg and X. Trepat, *Nat. Mater.*, 2011, **10**, 469.
7 X. Serra-Picamal, V. Conte, R. Vincent, E. Anon, D. T. Tambe, E. Bazellieres, J. P. Butler, J. J. Fredberg and X. Trepat, *Nat. Phys.*, 2012, **8**, 628.
8 M. Poujade, E. Grasland-Mongrain, A. Hertzog, J. Jouanneau, P. Chavrier, B. Ladoux, A. Buguin and P. Silberzan, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 15988.
9 C. G. Rolli, H. Nakayama, K. Yamaguchi, J. P. Spatz, R. Kemkemer and J. Nakanishi, *Biomaterials*, 2012, **33**, 2409.
10 S. R. K. Vedula, M. C. Leong, T. L. Lai, P. Hersen, A. J. Kabla, C. T. Lim and B. Ladoux, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 12974.
11 L. Q. Wan, K. Ronaldson, M. Park, G. Taylor, Y. Zhang, J. M. Gimble and G. Vunjak-Novakovic, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 12295.
12 M. Kamimura, O. Scheideler, Y. Shimizu, S. Yamamoto, K. Yamaguchi and J. Nakanishi, *Phys. Chem. Chem. Phys.*, 2015, **17**, 14159.
13 M. Reffay, M. C. Parrini, O. Cochet-Escartin, B. Ladoux, A. Buguin, S. Coscoy, F. Amblard, J. Camonis and P. Silberzan, *Nat. Cell Biol.*, 2014, **16**, 217.
14 T. Das, K. Safferling, S. Rausch, N. Grabe, H. Boehm and J. P. Spatz, *Nat. Cell Biol.*, 2015, **17**, 276.
15 E. Bazellières, V. Conte, A. Elosegui-Artola, X. Serra-Picamal, M. Bintanel-Morcillo, P. Roca-Cusachs, J. J. Muñoz, M. Sales-Pardo, R. Guimerà and X. Trepat, *Nat. Cell Biol.*, 2015, **17**, 409.
16 J. Nakanishi, Y. Kikuchi, S. Inoue, K. Yamaguchi, T. Takarada and M. Maeda, *J. Am. Chem. Soc.*, 2007, **129**, 6694.
17 S. Kaneko, H. Nakayama, K. Yoshino, D. Fushimi, K. Yamaguchi, Y. Horiike and J. Nakanishi, *Phys. Chem. Chem. Phys.*, 2011, **13**, 4051.
18 S. Kaneko, K. Yamaguchi and J. Nakanishi, *Langmuir*, 2013, **29**, 7300.

19 Y. Shimizu, H. Boehm, K. Yamaguchi, J. P. Spatz and J. Nakanishi, *PLoS One*, 2014, **9**, e91875.
20 R. S. Fischer, K. A. Myers, M. L. Gardel and C. M. Waterman, *Nat. Protoc.*, 2012, **7**, 2056.
21 R. Long, M. S. Hall, M. Wu and C. Hui, *Biophys. J.*, 2011, **101**, 643.
22 Y. Nagasaki, *Polym. J.*, 2011, **43**, 949.
23 S. Kidoaki and T. Matsuda, *J. Biotechnol.*, 2008, **133**, 225.
24 V. C. Shukla, N. Higuita-Castro, P. Nana-Sinkam and S. N. Ghadiali, *J. Biomed. Mater. Res., Part A*, 2016, **104**, 1182.