An Application of Photoactivatable Substrate for the Evaluation of Epithelial-mesenchymal Transition Inhibitors

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Epithelial-mesenchymal transition (EMT), phenotypic changes in cell adhesion and migration, is involved in cancer invasion and metastasis, hence becoming a target for anti-cancer drugs. In this study, we report a method for the evaluation of EMT inhibitors by using a photoactivatable gold substrate, which changes from non-cell-adhesive to cell-adhesive in response to light. The method is based on the geometrical confinement of cell clusters and the subsequent migration induction by controlled photoirradiation of the substrate. As a proof-of-concept experiment, a known EMT inhibitor was successfully evaluated in terms of the changes in cluster area or leader cell appearance, in response to biochemically and mechanically induced EMT. Furthermore, an application of the present method for microbial secondary metabolites identified nanaomycin H as an EMT inhibitor, potentially killing EMTEd cells in disseminated conditions. These results demonstrate the potential of the present method for screening new EMT inhibitors.

Keywords Collective cell migration, epithelial-mesenchymal transition, leader cells, cancer, drug screening, nanaomycin, patterning, caged compound, mechanobiology, transforming growth factor β

(Received September 29, 2018; Accepted October 23, 2018; Advance Publication Released Online by J-STAGE November 2, 2018)

Introduction

Epithelial-mesenchymal transition (EMT) is a biological process where adherent and polarized epithelial cells change into an individual migratory and nonpolarized mesenchymal phenotype (Fig. 1A).^{1,2} Together with its reverse reaction, mesenchymal-epithelial transition (MET), it is involved in various physiological processes, including, morphogenesis, tissue repair, and wound healing. Also, EMT triggers pathological processes, such as organ fibrosis and cancer progression.³ Especially, most cancer is developed in epithelial tissues, where cells gain out-of-control proliferative activities, followed by destruction of the basal membrane, invasion into the stroma, and metastasis through blood vessels to form secondary tumors in distal tissues. During these processes, EMT and MET are closely involved in the invasion and metastatic foci formation, respectively.⁴ These facts make EMT a new target for anticancer therapy.⁵ One of the unique characteristics of EMT as a screening target is its involvement of multiple extracellular mediators and signaling.⁶ This feature makes it difficult to evaluate EMT by isolated molecular targets, like receptors and enzymes, as commonly used for other types of drug screening.⁵ Therefore, to date, drug candidates targeting EMT are mostly evaluated based on phenotypical changes in cell lines,⁷ in terms of the expression

changes of epithelial or mesenchymal marker genes, or by evaluating the change in cellular migration behaviors in 2D or 3D substrates.⁸ In the conventional screening platforms, cells were first stimulated by soluble EMT inducers, such as transforming growth factor- β (TGF- β),⁹ epidermal growth factor (EGF),¹⁰ and hepatocyte growth factor (HGF),¹¹ thereafter the ability of drug candidates to retrieve the epithelial phenotypes were evaluated. However, there were almost no reports on the screening assays that can be used to identify compounds that inhibit EMT in response to other extracellular stimuli, such as ECM composition alteration and mechanical stress. Therefore, conventional screening methods are not suitable to identify compounds that regulate such environmental-cue-induced EMT signaling.

Cellular patterning is a technology for the spatial control of cell adhesion on the substrate surface.^{12,13} It is not only useful for the creation of tissue-mimicking heterotypic cell assemblies from the tissue engineering viewpoint, but also for controlling mechanical stress applied to the cells. The latter aspect is owing to the cellular endogenous force generation driven by the actomyosin contractility.¹⁴ An epoch-making study in this direction is the alteration of differentiation lineages of mesenchymal stem cells (MSCs) by changes in the degree of spreading at the single cell level.¹⁵ The same strategy can be applied to multi-cell clusters. In this case, however, mechanical stress in each cell is transmitted across intercellular junctions, resulting in selective higher tensile force applied to the cells located at the edge of clusters. Such non-homogenous force

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Fig. 1 Research concept of this study. (A) Cells actively change their phenotype between epithelial (yellow) and mesenchymal (green) through epithelial-mesenchymal transition (EMT) and *vice versa* (MET). (B) Selective induction of partial EMT in edge cells due to higher traction force. (C) The photocleavage reaction of the 2-nitrobenzyl (2-NB) group tethered with PEG. (D) Schematic representation of the photoactivatable gold substrate used in the present study. The gold surface was functionalized with PCP-ds, cRGD-ds, and EG₆-ds. The surface changes from non-cell-adhesive to cell-adhesive in response to near-UV irradiation ($\lambda = 365$ nm). (E) The procedure for the preparation of a geometrically controlled cell cluster and the migration induction. The photoactivatable substrate is irradiated through a photomask (1st UV) to form micropatterned cell adhesiveness on the surface. Cell seeding results in selective attachment of cells corresponding to the initial pattern. Selective irradiation of the remaining regions (2nd UV) releases the geometrical confinement and induces cell migration. The cluster expansion behavior changes depending on the EMT inhibitor candidates added at the last step of the procedure.

distribution across the clusters endows the edge cells with unique activities, such as higher proliferation activities¹⁶ and selective EMT induction (Fig. 1B).¹⁷ Therefore, micropatterned cells can be platforms for drug screening against mechanically-induced EMTed cells.

Our group has developed photoactivatable substrates bearing poly(ethylene glycol) (PEG) *via* photocleavable 2-nitrobenzyl (2-NB) group (Fig. 1C).¹⁸ The substrate surface change from non-cell-adhesive in response to near-UV irradiation (Fig. 1D), therefore cell clusters with arbitrary geometry can be prepared precisely and reproducibly by controlling the photoirradiation regions (Fig. 1E). In addition to the geometrical control of cell clusters, their collective migration behaviors can be analyzed by releasing the geometrical confinement by the secondary irradiation of initially non-adhesive surrounding regions. This feature enabled us to study the effect of cluster geometry,¹⁹ substrate adhesiveness,^{20,21} or stiffness²² on collective migration characteristics or to leader cells,²³ which actively produce lamellipodia and lead migration of cell collectives. Therefore, this platform will not only allow us to mechanically induce EMT to edge cells, but also analyze cluster expansion behaviors and the appearance of leader cells. To test this hypothesis, in this study, we first assessed how known EMT inducers and inhibitors affect cluster expansion behaviors of Madin-Darby canine kidney (MDCK) cells, a typical cell line used for EMT studies, by using photoactivatable gold substrates.²¹ Thereafter, we applied this methodology to naturally occurring compounds to identify potential EMT inhibitors.

Experimental

Reagents, chemicals, and photoactivatable substrates

Minimum essential medium (MEM) was purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from BioWest (Nuaille, France). Trypsin-ethylene-diaminetetraacetic acid (EDTA), penicillin-streptomycin, sodium pyruvate, dimethylsulfoxide (DMSO), L-glutamine, bovine serum albumin (BSA), and CultureSure® SB431542 (Wako) were obtained from Wako (Osaka, Japan). Recombinant human transforming growth factor- β 1 (TGF- β 1) was purchased from Funakoshi (Tokyo, Japan). Pyrizomicin A²⁴ and Nanaomycin H²⁵ were isolated from a culture broth of *Streptomyces rosa* subsp. *notoensis* OS-3966 and *Lechevalieria aerocolonigenes* K10-0216, respectively, by physicochemical screening.²⁶ Two disulfide compounds, photocleavable PEG (PCP)-ds and cRGD-ds, were synthesized based on the previous reports.^{27,28} EG₆-terminated disulfide (EG₆-ds) was purchased from ProChimia (Sopot, Poland). Gold substrates were prepared by vacuum deposition of a 5-nm titanium layer followed by a 15-nm gold layer on a glass cover slip (0.12–0.17 mm thick, Matsunami, Japan) with an E-beam evaporator. The substrate was cleaned by a UV-ozone cleaner (UV253, Filgen, Nagoya, Japan) and functionalized with cRGD-ds, EG₆-ds, and PCP-ds by placing an aliquot of their mixed methanolic solution (cRGD-ds:EG₆-ds:PCP-ds (v/v) as 0.0001:0.0999:0.9) at total concentration 50 μ M on the surface and left overnight at room temperature.

Fig. 2 Effect of known EMT-related compounds on cluster expansion behaviors of MDCK cells. (A–C) Representative images during expansion of a circular cluster in the presence of (A) 10 ng/mL TGF- β , an EMT inducer, (B) 10 μ M SB-431542, an EMT inhibitor, (C) 0.5% DMSO (control). Also, 0.5% DMSO was added to A and B. Time after secondary irradiation is shown. Arrowheads indicate leader cells. Scale bar: 100 μ m. (D) Relative cluster area change during 12 h. (E) Number of leader cells at 12 h. Data are means $\pm$ S.D ($n = 3$). Statistical analysis was evaluated using two-tailed Student's *t*-test.

Cell dynamic patterning

MDCK cells (RCB0995, RIKEN cell bank) were cultured in MEM containing 10% FBS, 100 units/mL penicillin, 100 μ g/mL streptomycin, 1% MEM-nonessential amino acids, and 1% sodium pyruvate, at 37°C, in a humidified atmosphere containing 5% CO₂. Cells were subcultured every 2–3 days by using trypsin-EDTA. Cells were patterned within a circular spot on the photoactivatable gold substrates placed in a glass-bottom dish (MatTek, MA, USA) and their migration was induced according to the procedure described in the previous report.²⁹ Each reagent was added to the dish just before induction of migration and their effect on cell migration behaviors were observed in a stage-top incubator (Tokai Hit, Fujinomiya, Japan) equipped on the above microscopes. Cell cluster area changes were evaluated by manually drawing the outline of cell clusters in the phase-contrast images by using Image J software.

Cell viability assay

MDCK cells were treated with 10 ng/mL TGF- β 1 plus 0.001% BSA, or 0.001% BSA alone, for 3 d. The cells were harvested by trypsin-EDTA and seeded at 2000 or 20000 cells/well density in a Falcon 96-well plate (Corning, NY, USA) and allowed to attach for 1 d. The cells were treated with 50 μ g/mL nanaomycin H containing DMSO (final concentration 0.5%) or DMSO only and incubated for 1 d. Finally, the cell viability was analyzed by MTS cell proliferation colorimetric assay kit (Funakoshi) according to the manufacturer's instructions.

Results and Discussion

In this study, we used a photoactivatable gold substrate developed in our previous work (Fig. 1D).²¹ The substrate surface was mixed self-assembled monolayers composed of three disulfide compounds bearing photocleavable PEG (PCP, Mw = 12k), cRGD, and EG₆.²¹ At the initial state, the cell-adhesive cRGD ligand is hidden under the umbrella of bulky

PEG brushes, therefore cells cannot interact with the adhesive ligand, thereby the surface is non-cell-adhesive (Fig. 1D, left). The cRGD ligand becomes accessible to cells only when the PEG group is cleaved by near-UV irradiation (Fig. 1D, right). Also, thanks to the presence of the bioinert EG₆ group, non-specific adsorption of proteins from medium is minimized, thereby the cell-substrate interaction is mainly mediated by the cRGD ligand after the PEG photorelease, not by physically adsorbed proteins derived from culture medium or cellular secretion.

The procedure for cell patterning and migration induction on the photoactivatable substrate is shown in Fig. 1E. In this study, we focused on the collective migration behaviors of normal epithelial MDCK cells from a circular cluster with *ca.* 140 μ m diameter and added test compounds during the last cell migration induction step. Figures 2A–2C show representative images of cluster expansion behaviors in the presence of a typical EMT inducer (TGF- β 1) and an inhibitor (SB-431542), together with DMSO as a control. TGF- β 1 is a cytokine that induces EMT upon binding to its receptor tyrosine kinase existing in the plasma membrane. Whereas, SB-431542 is a specific inhibitor of TGF- β superfamily type I receptors ALK4 (activin receptor-like kinase 4), ALK5, and ALK7.³⁰ When we applied TGF- β 1 (10 ng/mL) just before the secondary irradiation, extensive cluster expansion beyond the initial circular boundary was observed (Fig. 2A). In addition to the cluster area increase, several leader cells, with large lamellipodia extension, were frequently observed at the edge region of the cluster (Fig. 2A arrow heads). On the other hand, cluster expansion behaviors as well as leader cell formation was less active in the presence of SB-431542 (10 μ M, Fig. 2B) or in the control conditions (DMSO, Fig. 2C). To evaluate the difference quantitatively, relative changes in the cluster area during 12 h were compared (Fig. 2D). The relative cluster increase after the TGF- β 1 stimulation was significantly higher than that for the SB-431542 and DMSO treatment (Fig. 2D, TGF vs. SB or DMSO). These results indicate, even though the MDCK cells were treated with

Fig. 3 Effect of test compounds on cluster expansion behaviors of MDCK cells. (A, B) Representative images during expansion of a circular cluster in the presence of (A) 50 µg/mL pyrizomicin A plus 0.5% DMSO and (B) 50 µg/mL nanaomycin H plus 0.5% DMSO. (A) All the cells were dead. (B) Only the cells indicated by arrow heads were dying. Scale bar: 100 µm.

Fig. 4 Effect of nanomycin H on MDCK cells undergoing EMT by TGF- β 1 stimulation. (A, B) Morphological changes of MDCK cells upon EMT induction by TGF- β 1. Phase-contrast images of MDCK treated with (A) 0.001% BSA (control) and (B) 10 ng/mL TGF- β 1 plus 0.001% BSA for 3 d. (C) Effect of EMT induction (control and TGF) and cell seeding density (2000 vs. 20000 cells/well) on cytotoxicity of MDCK cells in response to 50 µg/mL nanaomycin H. Cell viability was evaluated by the MTS assay. The colorimetric responses were normalized against the cells treated with 0.5% DMSO (solvent of nanaomycin H) only for each condition. Data are means $\pm$ SDs ($n = 3$).

TGF for such a short time (12 h), the cells initiated to undergo EMT and their migration activity became more aggressive. On the other hand, when we compared the cluster area changes between SB-431542 and the control conditions (DMSO), the cluster expansion was slightly retarded for SB, but the difference was not significant from the statistical analysis (Fig. 2D, SB vs. DMSO). When we quantified the number of leader cells at 12 h, the values were significantly different between any two conditions among the TGF, SB, and DMSO treatments. Since leader cells can be considered as morphological transition to early mesenchymal traits,³¹ the decrease in the number of leader cells by the SB-431542 indicated its inhibitory action on mechanically-induced EMT. These results confirmed that the

effect of known EMT inducers and inhibitors can be evaluated on the photoactivatable substrates in terms of cluster area change and/or leader cell appearance.

In addition to the evaluation method described above, we unexpectedly found another possibility for the identification of potential EMT inhibitors by using the photoactivatable substrates. Two natural products from the Kitasato Microbial Library,³² pyrizomicin A and nanaomycin H, were tested, whose actions on EMT/MET were unknown. Our earlier studies demonstrated that pyrizomicin A showed antimicrobial activity,²⁴ whereas nanaomycin H did not express any notable antimicrobial activity.²⁵ In agreement with previous findings, an application of pyrizomicin A at 50 µg/mL completely killed MDCK cells

(Fig. 3A). On the other hand, when we applied 50 $\mu\text{g/mL}$ nanaomycin H in the culture medium, we observed selective killing of edge cells (Fig. 3B). By consideration of the fact that the edge cells tend to undergo early EMT by mechanical stimulus, we anticipated that nanaomycin H had a capability to selectively kill EMTed cells. Such activity is distinct from the inhibitory actions of conventional EMT inhibitors, like SB-431542 discussed above, that block EMT progression.⁵ To test this hypothesis, MDCK cells were reacted with 10 ng/mL TGF- β 1 for 3 d to biochemically induce EMT and the effect of nanaomycin H was evaluated by MTS assay (Fig. 4). The EMT induction by TGF- β 1 was confirmed biochemically by the increase in zinc finger E-box-binding homeobox 1 (ZEB-1), a typical EMT marker, in the mRNA and protein levels (data not shown). It can be also seen morphologically from microscopic observations (Figs. 4A and 4B). When the TGF-treated cells were seeded in a 96-well plate in the confluent state (20000 cells/well), the nanaomycin H treatment (50 $\mu\text{g/mL}$) slightly reduced the viability of the EMTed MDCK cells down to 76%. On the other hand, when the cell seeding density was reduced to 2000 cells/well, the cytotoxicity of nanomycin H was further enhanced for control and TGF-treated cells (Fig. 4C, 2000 cells). Especially, TGF-treated MDCK cells were hardly alive in such a low cell seeding density. The results are reasonable by considering that the cells were more isolated in the lower cell seeding density and the situations are close to those of the edge cells in the circular clusters (Fig. 3B). Notably, the capability of nanaomycin H to selectively kill the EMTed cells in low cell seeding density is suitable for anti-cancer drugs, as cancer cells undergoing EMT disseminate during the invasion process. Even though we need further analysis of the reaction mechanisms of nanaomycin H on the selective toxicity on EMTed cells, these results strongly support the usability of the present photoactivatable platform for the evaluation of compounds targeting EMT or MET.

In summary, we herein demonstrated applications of photoactivatable substrates for the evaluation of EMT/MET inhibitors. The ability of known EMT inhibitor, SB-431542, on EMTed cells were evaluated in terms of cluster area change as well as leader cell appearance. Moreover, applications of naturally occurring compounds on this system identified a new potential EMT inhibitor, nanaomycin H, which can selectively kill mechanically- and biochemically-induced EMTed cells. With the help of either an automated tracking technique tracking of cell clusters³³ or the Live/Dead assay, the present platform will be useful for screening of new drug candidates targeting EMT and MET.

Acknowledgements

This study was supported in part by the Japan Society for Promotion of Science, Kakenhi (No. 18H02010 and 18K19946). We thank to Prof. Kazuo Yamaguchi (Kanagawa Univ.) for the photocleavable disulfide molecule.

References

1. R. Kalluri and R. A. Weinberg, *J. Clin. Invest.*, **2009**, *119*, 1420.
2. S. Lamouille, J. Xu, and R. Derynck, *Nat. Rev. Mol. Cell Biol.*, **2014**, *15*, 178.
3. H. Acloque, M. S. Adams, K. Fishwick, M. Bronner-Fraser, and M. A. Nieto, *J. Clin. Invest.*, **2009**, *119*, 1438.
4. Y. Wu, M. Sarkissyan, and J. Vadgama, *J. Clin. Med.*, **2016**, *5*, 13.
5. F. Marcucci, G. Stassi, and R. De Maria, *Nat. Rev. Drug Discov.*, **2016**, *15*, 311.
6. G. Moreno-Bueno, H. Peinado, P. Molina, D. Olmeda, E. Cubillo, V. Santos, J. Palacios, F. Portillo, and A. Cano, *Nat. Protocols*, **2009**, *4*, 1591.
7. J. Johzuka, T. Ona, and M. Nomura, *Anal. Sci.*, **2018**, *34*, 1189.
8. M. Vinci, S. Gowan, F. Boxall, L. Patterson, M. Zimmermann, W. Court, C. Lomas, M. Mendiola, D. Hardisson, and S. A. Eccles, *BMC Biology*, **2012**, *10*, 29.
9. K. Arai, T. Eguchi, M. M. Rahman, R. Sakamoto, N. Masuda, T. Nakatsura, S. K. Calderwood, K.-i. Kozaki, and M. Itoh, *Plos One*, **2016**, *11*, e0162394.
10. Z. Qin, W. He, J. Tang, Q. Ye, W. Dang, Y. Lu, J. Wang, G. Li, Q. Yan, and J. Ma, *J. Cell Physiol.*, **2016**, *231*, 120.
11. J. Farrell, C. Kelly, J. Rauch, K. Kida, A. García-Muñoz, N. Monsefi, B. Turriziani, C. Doherty, J. P. Mehta, D. Matallanas, J. C. Simpson, W. Kolch, and A. von Kriegsheim, *J. Proteome Res.*, **2014**, *13*, 2874.
12. M. Théry, *J. Cell Sci.*, **2010**, *123*, 4201.
13. S. Funano, N. Tanaka, and Y. Tanaka, *Anal. Sci.*, **2017**, *33*, 723.
14. V. Vogel and M. Sheetz, *Nat. Rev. Mol. Cell Biol.*, **2006**, *7*, 265.
15. R. McBeath, D. M. Pirone, M. N. Celeste, K. Bhadriraju, and C. S. Chen, *Dev. Cell*, **2004**, *6*, 483.
16. C. M. Nelson, R. P. Jean, J. L. Tan, W. F. Liu, N. J. Sniadecki, A. A. Spector, and C. S. Chen, *Proc. Natl. Acad. Sci. U. S. A.*, **2005**, *102*, 11594.
17. N. Gjorevski, E. Boghaert, and C. M. Nelson, *Cancer Microenviron.*, **2012**, *5*, 29.
18. J. Nakanishi, *Chem. Rec.*, **2017**, *17*, 611.
19. C. G. Rolli, H. Nakayama, K. Yamaguchi, J. P. Spatz, R. Kemkemer, and J. Nakanishi, *Biomaterials*, **2012**, *33*, 2409.
20. Y. Shimizu, H. Boehm, K. Yamaguchi, J. P. Spatz, and J. Nakanishi, *Plos One*, **2014**, *9*, e91875.
21. Y. Shimizu, M. Kamimura, S. Yamamoto, S. A. Abdellatif, K. Yamaguchi, and J. Nakanishi, *Anal. Sci.*, **2016**, *32*, 1183.
22. M. Kamimura, M. Sugawara, S. Yamamoto, K. Yamaguchi, and J. Nakanishi, *Biomater. Sci.*, **2016**, *4*, 933.
23. A. A. Khalil and P. Friedl, *Integr. Biol.*, **2010**, *2*, 568.
24. T. Kimura, Y. Inahashi, H. Matsuo, T. Suga, M. Iwatsuki, K. Shiomi, Y. Takahashi, S. Ōmura, and T. Nakashima, *J. Antibiot.*, **2018**, *71*, 606.
25. T. Nakashima, T. Kimura, R. Miyano, H. Matsuo, T. Hirose, A. Kimishima, K. Nonaka, M. Iwatsuki, J. Nakanishi, Y. Takahashi, and S. Ōmura, *J. Biosci. Bioeng.*, **2017**, *123*, 765.
26. T. Nakashima, Y. Takahashi, and S. Ōmura, *Biochem. Pharmacol.*, **2017**, *134*, 42.
27. J. Nakanishi, H. Nakayama, K. Yamaguchi, A. J. Garcia, and Y. Horiike, *Sci. Technol. Adv. Mater.*, **2011**, *12*, 044608.
28. S. Marlar, S. A. Abdellatif, and J. Nakanishi, *Acta Biomater.*, **2016**, *39*, 106.
29. J. Nakanishi, "Methods in Cell Biology", ed. M. Piel and M. Théry, **2014**, Vol. 120, Chap. 7, Burlington, MA, 117.
30. S. K. Halder, R. D. Beauchamp, and P. K. Datta, *Neoplasia*, **2005**, *7*, 509.
31. S. Corallino, M. G. Malabarba, M. Zobel, P. P. Di Fiore, and G. Scita, *Front. Oncol.*, **2015**, *5*, 45.
32. Y. Takahashi and T. Nakashima, *Antibiotics*, **2018**, *7*, 45.
33. Y.-H. Chang, H. Yokota, K. Abe, C.-T. Tang, and M.-D. Tasi, *J. Med. Biol. Eng.*, **2017**, *37*, 18.