A facile assay of epithelial-mesenchymal transition based on cooperativity quantification of cellular autonomous motions

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Abstract

Epithelial-mesenchymal transition (EMT), a qualitative change in cell migration behavior during cancer invasion and metastasis, is becoming a new target for anticancer drugs. Therefore, it is crucial to develop in vitro assays for the evaluation of the abilities of drug candidates to control EMT progression. We herein reported on a method for the quantification of the EMT based on particle image velocimetry and correlation functions. The exponential fitting of the correlation curve gives an index ($\lambda$), which represents transforming growth factor (TGF)-$\beta$1-induced EMT progression and its suppression by inhibitors. Moreover, real-time monitoring of the $\lambda$ value illustrates a time-dependent EMT progressing process, which occurs earlier than the bio-chemical changes in an EMT marker protein expression. The results demonstrate the usefulness of the present method for kinetic studies of EMT progression as well as EMT inhibitor screening.

Keywords: epithelial-mesenchymal transition, particle image velocimetry, drug screening, mathematical analysis, cancer, cell migration.
Introduction

Cell migration is an essential biological activity involved in various physiological and pathological processes. In general, epithelial cells migrate collectively, keeping their contacts with surrounding cells, whereas mesenchymal cells migrate in single cells. However, in some spatiotemporally limited situations of life, such as early embryogenesis, tissue regeneration, and tumor progression, these cells actively switch between the two distinctly different migration phenotypes through epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) (Figure 1A).\(^1\) Especially, the loss of cohesiveness to surrounding cells and increased motility of epithelial cancer cells are critical steps for invasive cancer and metastasis, therefore, EMT is becoming a primary target of anticancer drugs.\(^2,3\) Either for the rapid screening of lead compounds or for the precise evaluation of the potency and efficacy of their structural derivatives, the methods to quantify the EMT progression are of essential importance. However, complex molecular changes during EMT\(^4\) make it difficult to evaluate EMT by isolated molecular targets, like receptors and enzymes, as commonly used for other types of drug screening.\(^2\) Therefore, EMT inhibitors have been mostly identified by phenotypically, in terms of the expression changes of epithelial or mesenchymal marker genes, such as E-cadherin and vimentin, or spheroidal expansion behaviors whether the cells preferred to stay as cell collectives or to spread out on 2D substrates.\(^5,6\)

In this study, we report on a facile method for quantification of EMT progression by mathematical analysis of a pair of successive phase-contrast images of cellular monolayers formed in normal culture dish without any need of biochemical analyses and sophisticated materials or devices. Figure 1 illustrates the quantification strategy. The method relies on particle-image velocimetry (PIV) commonly used for the analysis of flow field.\(^7\) Instead of the automatically tracking each cell,\(^8\) PIV treats a phase-contrast image as the aggregate of gray-scale pixels and the displacement field was obtained from the similarity between the successive two
images (Figure 1B,C). Each vector $\vec{a}$ of the displacement field is located on a square grid with a spacing of $\ell$ (Figure 1D). The spatial correlation function for each given discrete distance $r = k\ell$ ($k = 0,1,\ldots$) was calculated according to the following equations:

$$C(r) = \frac{1}{2} \sum_{m=x,y} \frac{\Sigma_i \vec{d}(\vec{r}_i) \cdot \vec{d}(\vec{r}_i + r\vec{e}_m)}{\Sigma_i \vec{d}(\vec{r}_i) \cdot \vec{d}(\vec{r}_i)} \quad (1)$$

where $\vec{e}_x = (1,0), \vec{e}_y = (0,1)$, $\vec{r}_i$ denotes the position of the $i$-th grid point, and the sums of each $i$ are taken over all grid points. The spatial correlation functions of the displacement field represent the degree of cooperativity of cellular monolayers, with more rapid decays in the correlation curves as losing cellular collective motion. Therefore, fitting the correlation function data $(r, C(r))$ for $r = k\ell$ ($k = 0,1,\ldots,10$) to an exponential function $\exp(-\lambda r)$ gives us an index $\lambda$, which becomes higher as decreasing in cellular cooperativity upon EMT progression.

Even though PIV and correlation functions (mostly, correlation distance, defined as the distance where $C(r) = 0$) have been commonly used for analyzing cellular cooperative motion in the directed migration mode, such as wound healing assay or cell migration along micropatterned adhesiveness, as far as we know, only a few studies, in early history, applied PIV and the correlative function to autonomous motion of confluent cellular layers, and specifically there was no report on their application to the EMT quantification. Therefore, we evaluated whether such mathematical approach can be used to represent the EMT progression in Madin-Darby canine kidney (MDCK) cells, a widely-used epithelial cell line in general EMT studies, in response to transforming growth factor (TGF)-$\beta 1$. 

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Experimental

Reagents and chemicals

All reagents were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), TCI Co., Ltd. (Tokyo, Japan), Sigma-Aldrich (St. Louis, MO, USA), Inc., unless otherwise stated.

Cell culture, EMT induction, and migration observation.

MDCK cells were grown in MEM supplemented with 10% heat inactivated FBS (Biowest, Nuallie, France), 1% MEM-NEAA, 1% sodium pyruvate, and 1% penicillin-streptomycin in 37°C with 5% CO2. The cells were subcultured every 2-3 days by using 0.25% trypsin-EDTA until use.

For EMT induction, the harvested cells (7.0 × 10⁵) were seeded in a 10-cm dish one day before and treated with a given concentration of human TGF-β1 (recombinant, PeproTech, Rocky Hill, NJ) containing 0.1% BSA (w/v) in the presence or absence of EMT inhibitors, i.e., CultureSure® SB431542 (Wako) or prostaglandin E2 (Sigma), for 2 d. The cells were harvested with trypsin-EDTA and seeded into a 6-well plate in a given density and un-attached cells were removed at 1–2 h after seeding. The cells were kept in the culture conditions in a stage-top incubator (Tokai hit, Fujimiya, Japan) under an inverted microscope, Axiovert 200 (Zeiss, Oberkochen, Germany). The observation was started at totally 3 h after seeding, which is defined as time = 0 throughout the study, and images were captured every 5 min through a 10× objective lens (Zeiss, NA = 0.30) by using a CCD camera (CoolSNAP MYO, Photometrics, Tucson, AZ) and a multi-well type motorized stage (Molecular devices, Downingtown, PA), both were con-trolled by MetaMorph Software (Molecular devices).

Immunofluorescence and cytoskeletal characterization

The cells were fixed by using 4% paraformaldehyde for 10 min followed by rinsing with PBS and quenching with 5% glycine. Then, the cells were permeabilized with 0.5 % triton X-100 in
PBS for 5 min at room temperature, and rinsed with PBS. The fixed cells were blocked with 5 % BSA for 1h, and then incubated overnight with mouse anti E-cadherin antibody (1:1000) at 4°C. Next day, the specimen was washed with PBS for three times, followed by incubation with Alexa Fluor 488-conjugated chicken anti-mouse (1:1000, ThermoFischer), Alexa Fluor 568 Phalloidin (1:1000, ThermoFisher). Finally, the sample was washed with PBS three times and covered with 50 % glycerol to avoid evaporation during observation.

**Western blotting.**

The cells were washed twice with ice-cold PBS and incubated in ice-cold radioimmunoprecipitation assay buffer (Nacalai Tesque, Kyoto, Japan) for 30 min. The scraped cell suspension was centrifuged at 13,000 g for 10 min at 4°C and the total protein concentration was determined with DC Protein Assay (Bio-Rad) using BSA as a protein standard. The lysate was mixed with an equal volume of 2× SDS sample buffer containing 20 mM 2-mercaptoethanol (Wako) and boiled at 95°C for 5 min. Proteins were separated by SDS polyacrylamide gel electrophoresis on a 10% Mini-PROTEAN® TGXTM Gel (Bio-Rad). The protein bands were transferred to a nitrocellulose mem-brane (0.2-μm pore size, Bio-Rad) for 1 h at 15 V. The membrane was blocked using 5% BSA in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h at room temperature and incubated with anti-ZEB-1 (1:1000, Bethyl), anti E-cadherin anti-body (1:2000), anti-GAPDH antibody (1:2000) for overnight at 4°C. After washing with Tris-buffered saline with TBST, the blots were incubated with HRP-conjugated secondary antibodies. The HRP signal was detected by chemiluminescence with a Super Signal West Dura (Thermo Scientific) and images were acquired on an IVIS imaging system using live imaging soft-ware, version 3.2 (Xenogen IVIS® Lumina II, Summit Pharmaceuticals, Tokyo, Japan).
Particle image velocimetry (PIV).

Particle image velocimetry (PIV)-like analysis that measures a displacement field between images in time was performed offline using our custom software written in MATLAB. In the analysis, the time interval between two images was set to be 50 min. Because maximum displacement between two successive images was overestimated to be 15 pixel (13.2 μm), we decided the size of the interrogation region and the search region to be 15×15 pixels (13.2×13.2 μm²) and 41×41 pixels (36.0×36.0 μm²), respectively. In order to obtain displacement vector field, the correlation between two successive images $R$ was calculated based on the direct cross-correlation method, which is defined by the following equation:

$$R(\vec{d}(\vec{r}_i)) = \frac{\sum{(f(\vec{r}_j)-f_m)(g(\vec{r}_j+\vec{d})-g_m)}}{\sqrt{\sum{(f(\vec{r}_j)-f_m)^2(g(\vec{r}_j+\vec{d})-g_m)^2}}} \ (2)$$

where $\vec{r}_i$ is the position of the center of an interrogation region, $f$ and $g$ represent 2D arrays of image intensity values of the interrogation region and the search region, respectively, whereas $f_m$ and $g_m$ are their mean image intensity. The sums in equation (2) are taken to cover all grid points, $\vec{r}_j$, of the entire interrogation region. The correlation $R$ takes a maximum value at $\vec{d}(\vec{r}_i) = \vec{d}_s(\vec{r}_i)$ in the search region. The sub-pixel order of displacement vector $\vec{d}$ is obtained by quadratic interpolation using the vector field with $\vec{d}_s$.

Results and Discussion

The successful EMT induction in MDCK cells by the TGF treatment for 2 d can be observed as the drastic morphological changes from polygonal appearance to spread shapes, together with the change in the location of actin from cortical to stress fibers (Figure 2A). Also, the acquisition of the mesenchymal phenotype was further confirmed from the decrease in the expression level of
E-cadherin, a typical epithelial marker, detected by immunofluorescence (Figure 2A) and western blotting (Figure 2B). However, as-prepared cells were not suitable for the mathematical analysis due to non-homogenous cell distribution across the culture plates (Figure S1A) and unequal cell densities caused by different proliferation rates between TGF-treated and non-treated cells (Figure S1B). We, therefore, harvested these cells and reseeded into new culture plates to standardize the analytical conditions. Figure S1C shows phase-contrast images of TGF-treated and non-treated (BSA) cells with different seeding densities. In the lowest seeding density ($5 \times 10^5$ cell/35-mm dish), cell-free regions were detected for both TGF-treated and non-treated cells, but it became almost undetectable when the cell seeding density was increased up to $1 \times 10^6$ cells/35-mm dish. Then, we manually counted the density of adhered cells from these phase-contrast images (Figure 2C). Because of the higher spreading property of the TGF-treated cells, the cell number was saturated at $1 \times 10^6$ cells/35-mm dish (Figure 2C, red circles), whereas the cell number continued increasing for non-treated MDCK cells due to the squeezing capability of epithelial cells (Figure 2C, black circles). We next examined the relationship between the density of attached cells and the lambda values calculated based on the strategy shown in Figure 1. In case of non-treated MDCK cells, the lambda gradually decreased and reached to a plateau value, indicating an initial increase in cell collectivity followed by its saturation at a certain cell density (Figure 2D, black circles). The lambda value for the TGF-treated cells showed a similar tendency, but with entirely higher value than the non-treated ones (Figure 2D, red circles), clearly reflecting the loss of collectivity through the EMT progression in response to TGF-$\beta$1. By consideration of cellular intrinsic proliferative activity during the measurements, it is crucial that the lambda value is not fluctuated by changes in cell density for the robust analysis. Therefore, we determined the seeding density of $1 \times 10^6$ cell/35-mm dish was suitable to start with. This feature was further verified by monitoring time profiles of lambda values for several hours. Throughout this measurement time, the lambda value for the TGF-treated cells kept higher than non-treated ones. Notably, even
though the morphological difference between the TGF-treated and non-treated cells became less obvious by reseeding compared to those before trypsin harvesting (Figure 2A vs. S1C), the mathematical indexes can clearly reflect the difference in their collective motions. When we looked into the time profiles more carefully, we noticed that the lambda values declined until initial 5 h, followed by relatively stable phase for next 10 h (Figure 2E). The initial decreasing phase can be attributed to the maturation of cell-cell cohesion, thereby increasing cell collectivity. Even though we can expect further maturation of E-cadherin-mediated cell-cell junctions by elongating culture time,\textsuperscript{16} those results indicate that 5 h is an appropriate time to represent the change in the autonomous motions of MDCK cells based on the lambda values.

We next examined whether the mathematically determined collective index can be used to evaluate the impact of EMT inhibitors. For this purpose, we chose two compounds, SB431542 and prostaglandin E\(_2\) (PGE\(_2\)), which react at different sites of signal transduction in TGF-\(\beta\)1-induced EMT. SB431542 is an inhibitor for TGF-\(\beta\) receptor 1, which directly blocks the starting point of TGF-\(\beta\) signaling.\textsuperscript{17} Whereas, PGE\(_2\) binds to the EP receptors and regulates the glycogen synthase kinase (GSK)-3\(\beta\), which eventually degrade Smad3, an essential mediator of TGF-\(\beta\)1-induced transcription.\textsuperscript{18} In our experimental conditions, MDCK cells treated with SB431542 (5 \(\mu\)M)\textsuperscript{19} completely restored their original epithelial phenotypes (Figure 3A, BSA vs. TGF+SB), whereas the treatment of PGE\(_2\) (1 \(\mu\)M)\textsuperscript{20} were less effective based on their spread morphology (Figure 3A, BSA vs. TGF+PGE) and stress fiber formation (Figure S2D). Regardless of the difference in their appearance, the expression level of Zinc finger E-box binding homeobox 1 (ZEB-1), an essential transcription factor in TGF-\(\beta\)1-induced EMT in MDCK cells,\textsuperscript{21} clearly represented TGF-\(\beta\)1-induced EMT progression and their blockage both by SB431542 and PGE\(_2\) (Figure 3B). Furthermore, the mathematically determined lambda values for these cells at 5 h after cell reseeding exhibited a similar tendency (Figure 3C) with a good linear relationship in between (Figure 3D). Therefore, we can conclude that the mathematically determined lambda
values was robust collectivity index for the EMT inhibitor screening. Interestingly, the lambda for the SB-treated cells were slightly lower than the non-treated ones. Similar decrease was observed in ZEB-1 expression, as well. By considering the regulation of epithelial plasticity in MDCK cells by the autocrine effect of TGF-β, these results indicate that, in addition to the application to EMT inhibitor screening, the present approach also has a potential for the evaluation of MET inducers. Also, it should be emphasized that the mathematical approach is extremely more rapid and simple than time-consuming western blotting analysis.

So far, a sufficiently high concentration of TGF-β1 (10 ng/mL) was used to absolutely induce EMT in MDCK cells. Next, we evaluated the capability of the mathematical approach to represent the intermediate states of EMT progression. For this, we first decreased the concentration of TGF-β1. The western blotting showed a gradual increase in the ZEB-1 expression level above 0.1 ng/mL TGF-β1, indicating the successful production of partially EMTed states in MDCK cells by these un-saturated doses of TGF-β1 (Figure 4A). When we evaluated the lambda value in these cells, it gradually increased as elevating TGF concentration (Figure 4B). Even though the detection limit (0.3 ng/mL), based on 3SD of blank, was slightly higher than that of the ZEB-1 expression level (0.1 ng/mL, Figure 4A), these results verified the capability of the lambda value to reflect the intermediate state of EMT.

Finally, we looked at the very beginning process of EMT acquisition. Here, we investigated the changes in cellular collective motions just after stimulation with TGF instead of using cells incubated with TGF for enough long time (2 d) as used so far. Non-treated MDCK cells were seeded in new culture plates, where 10 ng/mL TGF-β1 or vehicle (0.1% BSA) was added at 3 h after cell seeding (this time is defined as time = 0 in the graphs). As shown in Figure 2E, the lambda value showed a time-dependent decline due to the maturation of cell-cell cohesion, reaching to relatively stable phase in the control conditions (black profile, Figure 4C). On the other hand, when cells were treated with TGF-β1, the initial lambda decrease was smaller,
equilibrating into a significantly higher level than that of non-treated control (red line, Figure 4C). These results indicated less maturation of cell-cell cohesion due to the initiation of EMT progression process. Notably, within this time frame, there was no detectable difference in ZEB-1 expression determined from western blotting (Figure 4D) as well as E-cadherin distribution (Figure S3). Moreover, the present mathematical approach allowed us to evaluate the very early step of EMT acquisition, which suggests the potential application for the study of dynamics of EMT acquisition.

In this study, we report on an approach for the conversion of qualitative change of EMT into quantitative values based on PIV and correlation functions. The mathematically obtained lambda values demonstrated full and partial progression of TGF-β1-induced EMT and their blockage by EMT inhibitors in epithelial MDCK cells in good agreement with the expression level changes of a typical EMT marker, ZEB-1. Furthermore, the present method can be applied to indicate the initiation process of EMT, which cannot be detected by biochemical assays. Such feature highlights the advantage of the phenomenological approach and indicates its ability to find compounds that block the very early step of EMT. As the viewpoint of assay methods, the present strategy needs only two successive phase-contrast images with a given time interval in normal culture plates without any needs for specific reagents or devices. Such simple procedure is particularly advantageous for high-throughput screening of EMT and MET inhibitors, which is useful to regulate cancer metastasis and tissue fibrosis.

**Supporting Information**

Supporting Information showed the following. MDCK cells used for collectivity analysis (Figure 1S); F-actin distribution in MDCK cells treated with TGF together with inhibitors for 2 days in various conditions (Figure 2S); The difference in E-cadherin distribution in MDCK cells treated with TGF-β1 and vehicle (0.1% BSA) for 5 h (Figure 3S). This material is available free
of charge on the Web at http://www.jsac.or.jp/analsci/.

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**Figure Captions**

**Fig. 1** The concept of the present EMT quantification method. (A) Transition between epithelial and mesenchymal phenotypes through EMT and MET. (B, C) Schematic representation of the calculation strategy of displacement field by PIV. Phase-contrast images of a confluent cellular layer were divided into arrayed interrogate regions (red square). Each region was treated as an aggregate of gray-scale pixels (C), and most similar gray-scale patterns after interval $\Delta t$ (yellow square) was found from a search region (green square) larger than the interrogate region to obtain displacement vector at the lattice point. (D) A representative image of the cellular displacement field calculated by the PIV theory. (E) A representative graph of the correlation function of the displacement field. Its exponential fitting gives an EMT index, $\lambda$.

**Fig. 2.** Effect of TGF-$\beta$1 on the phenotype, adhesion, and migration of MDCK cell. (A) cell morphological, cytoskeletal and immunofluorescence characterization of EMT progression in MDCK cells stimulated by of 10 ng/mL TGF-$\beta$1 for 2 d. The cells treated with 0.1% (g/v) BSA are shown as control. (B) Western blotting results of E-cadherin expression levels with and without the TGF-$\beta$1 treatment. The data was normalized with that of the cells treated with BSA only. (C) The relationships between cell seeding density and cell adhered density for TGF-$\beta$1-treated (red) and non-treated cells (black) at 5 h of observation. (D) The dependence of lambda values on the cell adhered density for TGF-$\beta$1-treated (red) and non-treated cells (black) at 5 h of observation. (E) Time-dependent variation of lambda values in (red) the TGF-$\beta$1-treated and (black) the non-treated cells. The lambda value was started monitoring 3 h after cell seeding (time = 0). (B) Results of three independent experiments are shown as average ± S.D. (C-E) Plots represent average ± S.D. of four independent regions.

**Fig. 3** Validation of the lambda value for the evaluation of EMT inhibitors. (A) Phase-contrast
images of MDCK cells stimulated with 10 ng/mL TGF-β1 for 2 d in the presence of SB431542 (5 μM) or PGE₂ (1 μM). The images without the inhibitors and only with vehicle (0.1% BSA) were also shown as references. The images were taken before the cells were harvested from culture plates. (B) Evaluation of EMT inhibitions based on ZEB-1 expression determined by western blotting. Error bars represent standard deviations of three independent experiments. (C) Lambda values mathematically determined based on equation (1) shown in the text. Results of more than 14 independent regions from three to eight experiments are shown as a box plot. (D) Relationship between lambda value and the ZEB-1 expression level. The average values for B and C are plotted. The dotted line shows the linear fitting with R value shown in the graph. Scale bars indicate 25 μm.

Fig. 4  Application of the lambda value to represent intermediate states of EMT progression. (A) Western blotting results of ZEB-1 expression in MDCK cells treated with sub-saturated concentrations of TGF-β1 for 2 d. Error bars represent standard deviations of three independent experiments. (B) The lambda values for MDCK cells treated with various concentrations of TGF-β1 for 2 d. Plots represent average ± S.D. of eight different regions. (C) Evaluation of the onset of EMT acquisition, based on lambda values, in cells stimulated with (red) 10 ng/mL TGF-β1 and (black) vehicle (0.1% BSA). Non-stimulated MDCK cells were seeded in new culture plates and stimulated with TGF-β1 or vehicle, when cell observation started (time = 0). Error bars represent standard deviations for the values of six different regions. (D) Western blotting results of ZEB-1 expression for MDCK cells treated for 10 ng/mL TGF-β1 or vehicle (0.1% BSA) for 5 h.
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Graphical Index

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