Published in final edited form as:

Connect Tissue Res. 2018 July ; 59(4): 309–315. doi:10.1080/03008207.2017.1384471.

Contact Guidance and Collective Migration in the Advancing Epithelial Monolayer

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Abstract

At the edge of a confluent cell layer, cell-free empty space is a cue that can drive directed collective cellular migration. Similarly, contact guidance is also a robust mechanical cue that drives cell migration. However, it is unclear which of the two effects is stronger, and how each mechanism affects collective migration. To address this question, here we explore the trajectories of cells migrating collectively on a substrate containing micropatterned grooves (10–20 μm in periodicity, 2 μm in height) compared with unpatterned control substrate. Compared with unpatterned controls, the micropatterned substrates attenuated path variance by close to 70% and augmented migration coordination by more than 30%. Together these results show that contact guidance can play an appreciable role in collective cellular migration.

Introduction

Collective cellular migration of an advancing epithelial layer comes into play in morphogenesis [1], wound healing [1–3], and collective cancer invasion [4–7]. Cell migration can be directed by the cellular microenvironment, such as by aligned collagen fibers that characterize many connective tissues [1, 3, 4, 8–13]. More controlled conditions for study of cellular motions can be provided by defined grooved patterning of the cell

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substrate on the micron-nano scale in a process called contact guidance (CG) [2, 14, 15]. Although mechanisms of CG remain elusive [8, 16, 17], it is now well known that CG cues can drive the elongated shape of a single cell migrating in isolation [2, 14, 18, 19], fine-tune the differentiation abilities and functions of such a cell [20, 21], and promote a rapid cell migration [2, 16, 17, 22, 23]. However, the effects of CG on collective cellular migration and cellular cooperativity are not well defined [8, 17]. Moreover, empty space, such as a wound into which cells might migrate, is another fundamental cue that can trigger directed migration of the confluent cellular monolayer [12, 17, 24–29]. Here we report quantitative analysis of collective cellular migration into an empty space with and without CG cues. Using soft lithography to create microgroove patterns, we have studied the effect of CG on the migration of the advancing Madin-Darby canine kidney (MDCK) monolayer. Our results indicate that the combination of CG and empty space, compared with empty space alone, attenuates the path variance by 72% and augments the migration directionality and coordination by 36%. As such, CG causes collective cellular migration to become substantially more efficient.

Methods

Lentivirus production and transduction

In all experiments we used Madin-Darby canine kidney (MDCK) type II cells that were GFP labelled for the tight junction protein ZO-1. Lentiviruses were produced by co-transfection of HEK-293T cells in 6-well plate format. In brief, 12 μ l of FuGENE HD was mixed with 100 μ l of OptiMEM medium and was added to a mixture of 3 plasmids: 0.5 μ g of pCMV-VSV-G vector, 0.5 μ g of lentiviral packaging psPAX2 vector, and 1 μ g of the lentiviral expression vector pLN511. The mixture was incubated at room temperature for 20 minutes for complex formation. Suspended HEK-293T (1.8×10^6 cells) were added to each FuGENE HD/DNA complex tube, mixed well, and incubated for 5 min at room temperature before being added to a designated well in 6-well plate containing 1 ml cell culture medium, followed by incubation at 37°C with 5% CO₂. Media of transfected cells were replaced with 2.5 ml fresh culture media 18 hours post transfection. Supernatant containing newly produced viruses was collected at 48-hour post-transfection, and filtered through a 0.45 μ m syringe filter (Pall Corporation, Ann Arbor, MI; Catalog #4614). MDCK cells infected with pLN511 construct were sorted into high, medium, and low expressing level according to mEmerald intensity for subsequent analysis.

Cell culture

The MDCK cells were maintained in low-glucose Dulbecco's modified Eagle's medium (12320-032; Life Technologies, Carlsbad, CA) with 10% fetal bovine serum (Corning) and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO) in an incubator at 37°C and 5% CO₂.

Preparation of microgroove pattern

The grooved micropattern was created on a polydimethylsiloxane (PDMS) block. A well-mixed PDMS solution (base : curing agent = 10 : 1; Sylgard-184, Dow Corning) was poured on a microfabricated silicon mold with micropatterned grooves (periodicities of 10, 14, and

20 μm with a standard groove height of 2 μm) created by electron-beam lithography (MicroFIT, Seoul, Korea). The PDMS sample was cured by placing it on a hot plate (85°C) for 2 hours. The cured microfabricated PDMS was then removed from the mold and all patterned surfaces of the PDMS were coated with fibronectin (human plasma protein, Life Technologies). For a model wound assay [30], a half region (reservoir area) of the substrate was masked by a PDMS block before cell seeding. Then, MDCK cells were seeded (~80,000 cells) on the reservoir area ($6 \times 3 \text{ mm}^2$) and grown for 15 hours until the cells became confluent (Fig. 1).

Time-lapse fluorescence microscopy analysis

As the confluent layer advanced to fill cell-free space, we imaged cellular movements at 20 min intervals over 4 days using a 10 $\times$ objective lens (Leica Microsystems, Wetzlar, Germany). The imaging environment was maintained at 37°C /5% CO_2 in a heated enclosure (PeCon, Erbach, Germany).

Tracking cell migration and pathway analysis

Cell boundaries were clearly visible through a fluorescence filter, which was beneficial for cell tracking using the manual tracking method (ImageJ, NIH) with an analysis area of roughly $2 \text{ mm} \times 200 \mu\text{m}$ (Fig. 2a,b). A total of approximately 200 cells were analyzed from multiple independent experiments: 4 movies (1,300 min each) for non-CG substrate and 5 movies (800 min each) for CG substrate. For further quantification of the cell trajectories (Fig. 3), commercially available software was used (Chemotaxis and Migration Tool, Ibidi). We studied micropatterned plates with periodicities of 10, 14, and 20 μm . Data from different periodicities is analyzed individually as well as pooled together from all plates because periodicities between 10 and 20 μm did not show observable differences in cellular trajectories.

Results and Discussion

The micropatterned grooves and adjacent unpatterned control regions were used as substrates for cells from the same flat unpatterned cell reservoir. Movement of the cell monolayer was then triggered by lifting the PDMS block to expose the layer to a cell-free void (Fig. 1). The advancing cell monolayer migrated on either micropatterned grooved substrate (CG) or unpatterned substrate (non-CG). Time-lapse images showed that migration clearly differed between CG and non-CG regions. Migration speed was systematically higher on the CG substrate compared with the non-CG substrate—the time required to fill the open space (~1 mm) was 24 versus 72 hours respectively. In addition, fingering instabilities appeared at the leading edge in both systems, although the fingers in the CG regions were far sharper (Fig. 1c,d).

Trajectories of cells on the CG substrate compared with non-CG substrate were not only faster but also straighter (Fig. 2a,b). Because cell speeds between CG and non-CG substrates were systemically different, we compared trajectory shapes to quantify the effect of CG. We traced cellular trajectories over different time windows (800min for CG and 1,500min for non-CG) to achieve similar average end-to-end distance travelled between conditions. For

comparison across cells, initial cellular locations (x, y) of each trajectory were mapped to the origin (0, 0). For the non-CG substrates, most cell trajectories were strongly curvilinear and highly variable. For the CG substrate, by contrast, most cell trajectories were relatively straight and much less variable. As expected, net distance travelled in the x direction was not statistically different from zero in both conditions, although the x variability (*i.e.*, standard deviation of x at the end of trajectory, σ_x) was far smaller in the CG than in the non-CG case (7.29 versus 22.32 μm , $p < 0.005$). For the same average distance travelled, the y variability (*i.e.*, standard deviation of y at the end of trajectory, σ_y) was somewhat smaller in the CG than in the non-CG case (19.99 versus 25.47 μm , $p < 0.062$). The histogram of the angles between the origin and the end-points of each trajectory showed a narrower spread in the CG than the non-CG case. Overall, CG caused the x variance (σ_x) to decrease by 67% and the y variance (σ_y) to decrease by 21% (Fig. 2e,f).

To better understand the effects of the CG on the advancing cell monolayer, we quantified the cell trajectories in terms of the migration speed, path tortuosity, and X or Y forward migration indices, X_{fmi} and Y_{fmi} defined below (Fig. 2c,d). The migration speed was calculated from the displacements divided by the time interval in each step of the trajectories. The average migration speed was 1.4-fold faster in CG substrate than in the non-CG case ($p < 0.005$, Fig. 3a), perhaps as a result of cytoskeletal alignment along the micropatterns during cell migration [16, 17]. Interestingly, variability of migration speed (*i.e.*, standard deviation, σ_{speed}) was far larger in the CG substrate than in the non-CG case (0.05 versus 0.02 $\mu\text{m}/\text{min}$, $p < 0.005$). The larger deviation (σ_{speed}) of migration speed in the CG substrate compared with non-CG substrate was attributable to intermittent hesitation in forward movements and caterpillar-like contraction-elongation [31].

Next we quantified the path tortuosity which is defined as accumulated distance divided by Euclidean distance (Fig. 3b). As such, path tortuosity is a measure of the straightness of the trajectory; the closer the tortuosity is to 1, the straighter the trajectory is. The path tortuosity was close to 1 in the CG substrate (1.07 ± 0.03), whereas it was greater than 1 in the non-CG case (1.46 ± 0.07). This implies that trajectories were much straighter in the CG substrate than in the non-CG case, and is consistent with the histogram of the angular distributions showing a narrower spread in the CG (Fig. 2e,f).

The forward migration index (FMI) represents the preferred direction in the cell movements in 2D space:

$$X_{fmi} = 1/n \sum_{i=1}^n x_{i,end} / d_{i,accum}$$

$$Y_{fmi} = 1/n \sum_{i=1}^n y_{i,end} / d_{i,accum}$$

Here, $1 \leq i \leq n$ is the index of the cells (the first cell has the index 1, the last one n), x- or y-end is the final position of the trajectories at each time interval in the x and y-axis,

respectively, and d_{accum} is the accumulated distance. If a cell has equal probability of moving in the x-axis or y-axis, the larger the index of a given axis, the more preferred movements are along this axis. Because the cells headed to a cell-free void, the Y_{fmi} was higher than the X_{fmi} in both CG and non-CG cases (Fig. 3c). Moreover, Y_{fmi} was 1.3-fold larger in CG substrate than in the non-CG case ($p < 0.005$). These three metrics (the migration speed, path tortuosity, and FMI) were mutually consistent and, taken together, suggest that CG benefits the migration coordination by roughly 30~40%.

Conclusion

In summary, data presented here demonstrate that contact guidance exerts substantial effects on collective migration in the advancing epithelial monolayer. We used a model wound assay where the advancing monolayer is formed on micropatterned grooved substrate and compared with unpatterned control substrate. The cell migration trajectories were characterized by cell migration speed, path tortuosity, and forward migration index. We found that contact guidance cues can augment migration coordination by more than 30%, thereby remarkably attenuating the path variance (*i.e.*, angular distribution) by 72%. Further investigation of the cellular cooperativity in this system would provide information about the critical role of the contact guidance which might be linked to events such as morphogenesis, wound healing, and collective cancer invasion.

Acknowledgments

This work was funded by the National Cancer Institute grant number 1U01CA202123 and by the National Heart Lung and Blood Institute grant numbers R01HL107561-01, PO1HL120839, and T32 HL007118.

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Figure 1. Time sequence images showing the progress of confluent cell sheets on non- CG versus CG substrate

Images for (a,c,e) non-CG substrate and (b,d,f) CG substrate are taken from two separate culture dishes. Each image is a merge of the phase channel and the ZO1 fluorescence channel. Image size is $2 \times 1.3 \text{ mm}^2$. Images were taken at times; (a,b) $t = 0$, (c,d) $t = 720$ min, and (e,f) $t = 2,600$ min. $t = 0$ is set to the moment the PDMS block is removed.

Figure 2. Individual cell migration path during collective cell migration with and without contact guidance (CG) cues. (a and b)

Fluorescence images of ZO1-labeled MDCK cells in a confluent sheet on (a) non-CG versus (b) CG substrate. Image size, 2 mm × ~200 μm. The “comet tail” indicates the path taken, and the colors are assigned randomly. **(c and d)** The initial point of the trajectories are centered; (c) non-CG which was recorded for 1,300 min and (d) CG substrate which was recorded for 800 min (see Methods for detail). End point of each trajectory is colored red. Yellow cross markers on the cloud of end points indicates mean ± standard deviation. **(e and f)** Angular distribution of cell trajectories for the two substrates. The angle between the initial and the last positions of the individual cells is calculated from the trajectories displayed in (c) and (d), respectively.

Figure 3. Migration coordination and path variance were quantified by cell trajectory analysis Cell migratory measurements for (a) cell migration speed, (b) path tortuosity, and (c) X and Y forward migration indices (X_{fmi} and Y_{fmi}).