Facilitation of uniform maturation of human retinal pigment epithelial cells through collective movement in culture

Rie Sonoi, Mee-Hae Kim, and Masahiro Kino-oka*

Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka,

Suita, Osaka 565-0871, Japan

*Corresponding author

Tel: +81-(0)6-6879-7444; fax: +81-(0)6-6879-4246

E-mail address: kino-oka@bio.eng.osaka-u.ac.jp (M. Kino-oka)

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Footnote: Much of this work forms the basis of the Ph.D. dissertation of Rie Sonoi.

ABSTRACT

Understanding of the fundamental mechanisms that govern tight junction formation of retinal pigment epithelial (RPE) cells provides surface design strategies for promoting their maturation in culture. RPE cells were cultured to investigate their migratory behavior and the expression of tight junction protein ZO-1 in the central and peripheral regions of a culture vessel. Regardless of locational differences in the culture vessel, the cells at day 1 were elongated in shape, did not form tight junctions, and migrated actively. As the culture progressed, the cells in the central region slowly moved with morphological change of a cobblestone-like shape via interaction between contact cells and exhibiting the shift from random migration to collective movement toward the center, accompanied by tight junction formation. On the other hand, the cells in the peripheral region maintained the random migration at day 5, meaning spatial heterogeneity in maturation in the vessel. At day 5, RPE cells were incubated in medium with Rac1 inhibitor and the exposure to the Rac1 inhibitor triggered the rapid conversion of migratory behavior from random migration to collective movement toward the center of the vessel, resulting in uniform maturation. These findings indicate that the change in migratory patterns is an important cues and the collective movement toward the center causes the facilitation of uniform maturation in the vessel.

[**KEYWORDS:** Human retinal pigment epithelial cells, Maturation, Collective movement, Local heterogeneity, Confluence]

INTRODUCTION

Retinal pigment epithelial (RPE) cells form a part of the blood–retinal barrier and play a pivotal role in maintaining photoreceptor functions and local cellular homeostasis (1). Failure of these functions causes degeneration of the retina and loss of visual function (2). For the recovery of visual function, recent research has been dealing with clinical applications related to the transplantation of RPE cells derived from embryonic stem cells (ES) and induced pluripotent stem (iPS) cells (3-7). The preparation of RPE cells as graft material, entails two stages, namely, expansion and maturation processes. Through the maturation process in confluent culture, RPE cells acquire the typical phenotype through a morphological change from elongated to cobblestone-like shape, resulting in tight junction formation by expressing ZO-1 protein (8, 9). Further maturation leads to expression of RPE65 and CRALBP accompanied by Na^+ , K^+ -ATPase pump, and to expression of MITF with pigmentation (10–12).

Vegler et al. (12) reported that confluent human RPE cells derived from ES cells had the difference in expression patterns of the markers for RPE cells such as RPE65 and MITF from the center to periphery in the monolayer. In confluent culture, the migratory behavior of epithelial cells is altered in response to cell–cell adhesion level (13, 14), suggesting that the initiation for RPE maturation is influenced by their migratory behavior in a culture vessel. The assembly and disassembly of the actin cytoskeleton are further regulated by RhoA, Rac1, and Cdc42 of the Rho small GTPase family (15). Rac1 activation is required to promote actin polymerization and initial cell–cell contact (16, 17), while RhoA and Cdc42 are essential for maintaining cell–cell adhesions. In addition, RhoA activity is an antagonistic toward Rac1 activity (18-20). In a previous study, the

confluent cultures were conducted to understand the profiles of RPE cells in the initial state of maturation process and the migration affected spatial heterogeneity of tight junction formation in the vessel (21). In the present study, the spatio-temporal analysis for migratory behavior was further conducted to clarify the initiation of maturation of the cells in the vessel, and the uniform maturation was attempted.

MATERIALS AND METHODS

Culture conditions of human RPE cells Viable human RPE cells (Lot no. 0F3292; Lonza, Walkersville, MD, USA) were seeded at 5.0×10^4 cells/cm² on laminin-coated 48-well plates (culture area in each vessel: 0.95 cm²; diameter: 5.5 mm; Corning Constar, Cambridge, MA, USA) according to a method described previously (21). The laminin-coated surface was prepared by applying a solution of laminin-1 (Sigma-Aldrich, St. Louis, MO, USA) on the bottom surface of 48-well plates at 2 µg/cm². The cells were incubated for 5 days in RPE growth medium (One Retinal Pigment Epithelial Cell Medium BulletKit™; Lonza) at 37 °C in a humidified 5% CO₂ incubator, and the medium was changed every 2 days. The subsequent cells were exposed to Rac1 inhibitor NSC23766 (50 µg/ml; Calbiochem, Merck, Darmstadt, Germany) for 12 h. Culture time, t , was counted from the addition of the Rac1 inhibitor onwards.

Quantitative analyses of migratory behavior and maturation The schematic outline in Fig. 1 shows the procedure of data analysis for the migration rate and direction. Time-lapse images were captured to investigate cell behavior using an image analyzer with a 10× objective lens (IN Cell Analyzer 2000; GE Healthcare, Buckinghamshire, UK). The images were obtained from triplicate samples in 48-well plates, and original images (1.5 mm × 1.5 mm) at 16 bits in gray scale and 1.83

Fig. 1

pixels/ μm^2 resolution were captured every 20 min for 12 h on the entire surface. Multiposition capturing provided tiling images for all cells over the entire surface. For measurement of migration rate at the region of interest (ROI; $300\ \mu\text{m} \times 300\ \mu\text{m}$), the center of the round culture vessel was set at the central position by using the tiling images, and the edge of the vessel was defined as the position to be 5.5 mm from the center position. Data were obtained from the four ROIs placed at distance from the central position of the culture vessel, $r = 0, 1.5, 3.0,$ and $4.5\ \text{mm}$ (Fig. 1). The positional centroids (x_i, y_i) of each cell in the ROI were determined by using the image processing software (LabVIEW software; National Instruments, Austin, TX, USA) as described previously (21). The migration rate, V , of individual cells was determined from the displacement of positional centroids for 12 h. As shown in Fig. 1B, the angle between the vectors from the positional centroid of the cell to the center position of the vessel at 0 and 12 h after a certain measurement time, θ , was estimated ($0 \leq \theta \leq \pi$). The average migration rate toward the center, $\bar{V}_r$, was defined as the mean of $V_r = V\cos\theta$, which corresponds to the migration rate toward the center of the vessel. Data were obtained from 300–800 cells in triplicate cultures.

To assess tight junction formation, ZO-1 and cell nuclei were immunostained according to a previously described method (21). In brief, cells from the culture were washed, with PBS were fixed with 4% paraformaldehyde for 15 min at $4\ ^\circ\text{C}$, and permeabilized by incubation for 5 min in 0.2% Triton X-100. The cells were blocked by treatment with Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) for 1 h, and treated overnight with anti-rabbit ZO-1 antibody (Abcam, Cambridge, MA, UK) at $4\ ^\circ\text{C}$. After the cells were washed with Tris-buffered saline (TBS, Dako, Glostrup, Denmark), they were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG

(Life Technologies Corporation, Carlsbad, CA, USA) for 1 h. For nuclear staining, after a TBS, wash, the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Life Technologies Corporation) for 20 min at room temperature. Fluorescence signal intensities for ZO-1 and DAPI were obtained by excitation at the corresponding wavelengths of 488 and 358 nm, respectively. The staining images of ZO-1 and nuclei were captured using the image analyzer with a 10× objective lens.

To ascertain and quantify the association between maturation and migratory behavior, ZO-1 immunostaining images were captured after time-lapse observation in the same positions where migration rates were analyzed. The cells were divided into two groups, namely, ZO-1-positive and ZO-1-negative groups, according to the criteria depicted in Fig. 1C. The frequencies of ZO-1-positive and ZO-1-negative cells, F_Z and F_N , respectively, were estimated. In addition, the ZO-1-positive and ZO-1-negative cells were assumed to exhibit the collective movement and random migration, respectively. Thus, the frequency of cells with collective movement and random migration is equivalent to be F_Z and F_N , respectively. Furthermore, the frequency of cells with collective movement toward the center, F_{Zc} , was determined to be the frequency of ZO-1-positive cells migrating toward the center of the vessel ($V_r > 0$).

Statistical analysis All data were obtained from triplicate cultures, and were expressed as means with standard deviations. Comparisons between the groups were determined by one-way analysis of variance and the Tukey–Kramer post-hoc test. p values < 0.05 were considered statistically significant.

RESULTS

Maturation of RPE cells in culture

Spatio-temporal analysis was carried out in the central and peripheral regions at $r = 0$ and 4.5 mm, respectively. Immunostaining images of ZO-1 and cell nuclei were captured at the same positions. After seeding, the cells in both regions exhibited elongated shapes and migrated actively (Movie S1). As culture time elapsed, the cells in the central region shrank, and their random migration decreased. At day 5, the cells in the central region made a cobblestone-like shape, and they moved collectively with maintaining cell-cell contact. In contrast, the cells in the peripheral region at day 5 maintained the elongated shape and random migration, and the morphological change from elongated to cobblestone-like shape in the peripheral region was delayed. In both regions, positive ZO-1 expression was found in cells exhibiting collective movement. Thus, it is considered that the migratory behaviors of ZO-1-positive and ZO-1-negative cells were distinguished to be collective movement and random migration, respectively.

Effect of migratory inhibition on maturation

The timing of inhibition for migration was determined based on the data obtained from temporal analysis (Fig. 2). As the culture proceeded, it was found that the absolute values, $|V_r|$, decreased in the central region. At day 3, the ZO-1-positive cells with collective movement increased in the central region, although ZO-1-negative cells with active random migration are major in the peripheral region. At day 5, ZO-1 positive cells with collective movement were major ($F_Z = 0.75$) and the distribution is in the range of $V_r > 0$ in the central region, meaning that cells made collective movement toward the center in the vessel. On the other hand, cells in the peripheral region showed lower F_Z level and

Fig. 2

higher $|V_r|$ level, meaning the exhibition of collective movement and active random migration. To make detail clarification, the effect of migration behaviors on the initiation of maturation, the Rac1 inhibitor was added at day 5 ($t = 0$), and their migration rates and the directions in the central and peripheral regions from $t = 0$ to $t = 12$ h were examined.

As shown in Fig. 3, cells migrated slowly through interaction between neighbor cells, which had ZO-1 expression, under both conditions (Fig. 3a and b). In the culture without the Rac1 inhibitor, the migration rates of the cells in the peripheral region were higher than those in the central region, and cells migrating randomly and actively had negative ZO-1 expression (Fig. 3c), showing spatial heterogeneity for initiation of maturation. In the culture with the Rac1 inhibitor, the random migration of the cells in the peripheral region decreased and became unidirectional movement toward the center of the vessel, and their ZO-1 expression was positive, similar to the central region (Fig. 3d).

Fig. 3

The frequencies of cells with collective movement and migration rate toward the center were assessed along with radius direction (Fig. 4). At $r = 0$ in culture without the Rac1 inhibitor, the average migration rate was $\bar{V}_r = 0.0 \pm 1.7 \mu\text{m/h}$ (Fig. 4a). The standard deviations at $r = 0\text{--}4.5$ mm in culture without the Rac1 inhibitor increased with increasing the distance from the center of the vessel (Fig. 4a–d). At $r = 4.5$ mm, ZO-1-negative cells increased and the broad distribution of V_r were observed ranging from $V_r = -8.0 \mu\text{m/h}$ to $V_r = 8.0 \mu\text{m/h}$ (Fig. 4d), causing random migration. This means that spatial heterogeneity of migratory behavior exists in the vessel. In the culture with the Rac1 inhibitor (Fig. 4e–h), the distributions in V_r at $r = 0\text{--}4.5$ mm were significantly more narrow than those in culture without the Rac1 inhibitor (Fig. 4a–d). Especially, the standard

Fig. 4

deviation of V_r at $r = 4.5$ mm in the culture with the Rac1 inhibitor (Fig. 4h) was one fifth lower than that of the culture without the inhibitor (Fig. 4d). In addition, under both conditions, the frequency in cells with collective movement and $V_r > 0$ is more than half, meaning that the migration toward the center were caused by collective movement.

For understanding of the effect of collective movement toward the center on the maturation, the frequency of cells with collective movement toward the center, F_Z and F_{Zc} at $r = 0, 1.5, 3.0$, and 4.5 mm in the vessel at $t = 12$ h were compared. As shown in Fig. 5, F_Z and F_{Zc} at the central region in culture without Rac1 inhibitor was the highest ($F_Z = 0.75 \pm 0.10$ and $F_{Zc} = 0.71 \pm 0.14$), respectively, and these values decreased toward the periphery. Moreover, the difference between values of F_Z and F_{Zc} increased along with radius direction, being $F_Z = 0.47 \pm 0.10$ and $F_{Zc} = 0.21 \pm 0.10$ at $r = 4.5$ mm. In culture with Rac1 inhibitor, F_{Zc} at $r = 4.5$ mm was 3.5-fold higher than that of culture without Rac1 inhibitor, and F_{Zc} reached high level of 0.72–0.78 in whole vessel, meaning uniform maturation in the vessel. These results revealed that the migratory inhibition enhanced the tight junction formation with rapid conversion of random migration to collective migration, facilitating the movement toward the center as well as maturation.

Fig. 5

DISCUSSION

Migratory behavior is known to change in response to cellular phenotype, and the migratory behavior of individual RPE cells has been shown to be closely related to tight junction formation associated with their maturation (21). In the present study, time-lapse observation and immunostaining realized retrospective analysis of maturation of RPE cells. Immature RPE cells

1 cultured on laminin-coated surfaces were found to be elongated and exhibited random migration in
2 the confluent state (Movie S1). As shown in Fig. 6, this random migration is considered to occur Fig. 6
3 through the formation of lamellipodia at the leading edge of the cells. RPE cells cultured on
4 laminin-coated surfaces secrete fibronectin, and integrin $\alpha 5 \beta 1$ binding to fibronectin is the critical
5 step involved in cell migration and growth (22). Danen et al. (23) reported that the binding of
6 integrin $\beta 1$ to fibronectin increased Rac1 activity by autophosphorylation of focal adhesion kinase
7 (FAK), causing random migration by the formation of lamellipodia. These reports indicate that the
8 binding of integrin to fibronectin induces Rac1 activation by the autophosphorylation of FAK in
9 immature cells, which explains the random migration observed in the present study. During the
10 development of the adhesions between cells, their expression of ZO-1 increased, and their random
11 migration decreased. RPE cells generally express N-cadherin as their dominant cadherin (24, 25),
12 and p120 catenin binding to the cadherin localized to the cell membrane is known to cause
13 stabilization of cell–cell adhesions by suppressing endocytosis (26, 27). Tyrosine phosphorylation
14 of p120 catenin can induce the unbinding of cadherin complexes on the cell membrane (28), and
15 then the cytosolic p120 catenin is complexed with RhoA-GDP, thereby inhibiting RhoA activation
16 (29). Furthermore, RPE cells transfected with p120 catenin siRNA increase RhoA activity (30),
17 suggesting that the localization of p120 catenin to cadherin led to the increase in RhoA activity in
18 the present study. RhoA activation is known to decrease Rac1 activity (31) and induce myosin
19 activation by phosphorylating the light chain of myosin II, which leads to the assembly of tight
20 junction proteins (32). Considering these findings, it is most likely that RhoA activation caused the
21 assembly of tight junctions in our study. As maturation progresses, the actomyosin contraction

1 network and ZO-1 proteins assemble at the apical sides (33, 34), and RhoA activation increases
2 actomyosin contraction (19, 35). These cells are found to move collectively while exhibiting the
3 cobblestone-like shape (Movie S1). The cobblestone-like cells are supposed to increase dark
4 pigmentation, because microphthalmia-associated transcription factor induces the expression of the
5 tyrosinase gene controlling pigment synthesis (11, 36). Considering these findings, it can be stated
6 that the mature cells had migratory behavior, with the maintenance of cell–cell adhesions, which is
7 named as ‘collective movement’ (37, 38). Simultaneous actomyosin contraction in whole vessel is
8 considered to cause collective movement toward the center. These suggested that the transition from
9 random migration to collective movement is an important event to initiate the maturation.

10 Location-dependent analysis from the center toward the periphery of the vessel revealed that
11 the extent of the initiation for maturation gradually decreased along this path, and that the
12 frequencies of the cells having random migration increased at the same time (Fig. 4 and 5),
13 suggesting that the random migration in the vessel is responsible for locational heterogeneity of the
14 maturation in culture. In the present study, the Rac1 inhibitor was applied to suppress the random
15 migration of the cells in the vessel, and the cells in whole vessel slowly moved toward the center of
16 the vessel, although the inhibition of Rac1 actively had no effect on the initiation of maturation
17 against the cells already exhibiting collective movement (Fig. 5). The frequency of the maturation
18 in culture with Rac1 inhibitor, F_{Zc} , increased, similarly to that at the center. This uniform
19 maturation is considered to occur owing to the change in migratory behavior from random
20 migration to collective movement toward the center in the peripheral region. As illustrated in Fig. 6,
21 the inhibition of lamellipodium formation through suppression of Rac1 activity was curbed the

1 random migration, leading to the stabilization of cell–cell adhesions. Subsequently, RhoA activation
2 appears to have induced the assembly of tight junction proteins, and the cells in the periphery
3 reached a greater frequency of the maturation similarly to the cells at the center of the vessel. Taken
4 together, our findings reveal that suppression of random migration can facilitate switching to
5 collective movement toward the center, causing uniform maturation in the vessel.

6 In conclusion, RPE cells were cultured to characterize the location-dependent profiles of the
7 migratory behavior governing their maturation in culture. Time-lapse observation and ZO-1
8 immunostaining at the same positions where migration was analyzed were performed to examine
9 the association between migration and maturation. ZO-1-negative cells exhibited elongated shape
10 with random migration, while ZO-1-positive cells exhibited cobblestone-like shape with collective
11 movement. The migratory behavior of the random migration and collective movement implied
12 locational heterogeneity of maturation in the vessel. The suppression of migration resulted in the
13 facilitation of uniform maturation by collective movement toward the center. The present study
14 suggests that conversion of migration into collective movement toward the center is critical for
15 regulating the maturation of the cells in the vessel.

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Figure legends

FIG. 1 Procedure of data analyses for the directional migration rate of the cells at each region of interest (ROI) in a culture vessel.

FIG. 2 Frequencies of cells with collective movement and random migration against the migration rate toward the center of individual cells in the vessel at day 1 (a and d), 3 (b and e), and 5 (c and f). Closed and open bars show frequencies of cells with collective movement and random migration, respectively. The migration rate toward the center, V_r , of individual cells was determined from the displacement of positional centroids for 12 h. The average migration rate, $\bar{V}_r$, with standard deviation was calculated from the data obtained from 300–800 cells in triplicate cultures.

FIG. 3 ZO-1-negative and ZO-1-positive cells with their migration rate and direction in culture without (a and c) or with the Rac1 inhibitor (b and d) at $t = 12$ h ($300 \mu\text{m} \times 300 \mu\text{m}$ fields of view). The direction having $0 \leq \theta < \pi/2$ and $\pi/2 \leq \theta \leq \pi$ were considered migratory behavior toward the center (yellow vector) and periphery (red vector), respectively, of the vessel. The green and blue circles show ZO-1-negative cells with random migration and ZO-1-positive cells with collective movement, respectively. The green or blue circles without arrows depict cells with a migration rate of $V = 0.0$. The scale bars show $50 \mu\text{m}$.

FIG. 4 Frequencies of cells with collective movement and random migration (F_Z and F_N , respectively) against migration rate toward the center at $r = 0$ (a, e), 1.5 (b, f), 3.0 (c, g), and 4.5 mm (d, h) in culture without Rac1 inhibitor (a–d) and with the Rac1 inhibitor (e–h). Closed and open bars show frequencies of cells with collective movement and random

1 migration, respectively. The average migration rate and standard deviation were calculated
2 from the data obtained from 700–800 cells in triplicate cultures at day 5.

3 FIG. 5 Frequencies of cells with collective movement and with collective movement toward the
4 center (F_Z and F_{Zc} , respectively) at day 5. Open and closed circles show F_Z values in culture
5 without or with the Rac1 inhibitor, respectively. F_Z and F_{Zc} in each region were obtained
6 from three independent experiments ($n = 3$). Statistical significance among the groups was
7 determined by one-way analysis of variance (ANOVA) and the Tukey–Kramer post-hoc
8 test (* $p < 0.05$, ** $p < 0.01$).

9 FIG. 6 Schematic diagram of possible mechanism of the maturation based on migratory behavior of
10 RPE cells in culture.

Figure 1

Procedure of data analyses

A. Multiposition capturing of ZO-1 and bright-field images

C. Judgment of ZO-1-positive and ZO-1-negative cells

B. Assessment of cells migrating toward the central or peripheral position

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6