Locational Heterogeneity of Maturation by Changes in Migratory Behaviors of Human Retinal Pigment Epithelial Cells in Culture

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Running title: Maturation and migration of retinal cells in culture

Abstract

To better characterize human retinal pigment epithelial (RPE) cells, their maturation was studied by time-lapse observation and immunostaining of the tight junction protein ZO-1. During subconfluency with active migration, the cells had an elongated shape. During cell division to reach confluency, RPE cells became small and tight, exhibiting cobblestone-like morphology. In addition, RPE maturation at the peripheral region of the culture vessel was delayed when compared with the central region, demonstrating local heterogeneity during maturation. To correlate cellular migration and maturation, we compared frequencies of migration rate and number of ZO-1-positive cells at the central and peripheral regions. Cells having migration rates less than 5.0 $\mu\text{m/h}$ in the central region were 1.4-fold higher than in the peripheral region at day 5. Regardless of locational differences in the culture vessel, the frequency of cells having migration rates less than 5.0 $\mu\text{m/h}$ showed 90% agreement with the frequency of ZO-1-positive cells. To inhibit cell migration, RPE cells were exposed to medium containing 50 $\mu\text{g/ml}$ Rac1 inhibitor at day 5. Frequencies of ZO-1-positive cells and cells having migration rates less than 5.0 $\mu\text{m/h}$ at the peripheral region were similar to those at the central region. The results show that migration is an important factor affecting maturation, and demonstrate that location heterogeneity during maturation is caused by different migratory behaviors in the culture vessel.

[Key words: Human retinal pigment epithelial cells, Cell migration, Animal cell culture, Confluent state, Heterogeneity]

INTRODUCTION

Retinal pigment epithelial (RPE) cells, located between photoreceptors and the choriocapillaris, play a key role in maintaining normal vision, by transporting nutrients from the vascular choroid, by the formation of the blood-retinal barrier, and by absorption of scattered light (1). Because the retina is responsible for light detection, loss of this function due to damage or disease leads to the onset of blindness (2). One possible treatment for this loss is the replacement of damaged RPE cells with healthy cells. However, because of a limited supply of donated RPE cells, it is necessary to find alternative sources for transplantation (3, 4). Many groups have recently reported basic and clinical researches concerned with transplantation of RPE cells derived from embryonic stem cells and induced pluripotent stem cells (5-8). However, there are few reports focusing on analyses for understanding the maturation process of cultured RPE cells (9).

RPE cells have been cultured in the confluent state on substrates coated with an extracellular matrix to maintain monolayers with tight junctions (10). These mature RPE cells in the confluent state exhibited a cobblestone-like morphology (11, 12). Most techniques to evaluate the maturation of RPE cells involved genetic analyses as well as morphological observation with cell staining (13, 14). Immunostaining, when combined with fluorescent probes, can be used for visualization and quantification, and can be used as an imaging tool for characterization of the maturation state of individual cells during *in vitro* culture. However, it is still difficult to apply invasive methods for quantitative measurement of changes in the maturation state of RPE cells in culture.

In a previous study, based on analysis of local nuclear density, we described methodology to

1 estimate the early-state maturation of RPE cells on laminin-coated and plain surfaces (9). It was
2 suggested that laminin-coated surfaces could maintain monolayers of RPE cells at high nuclear
3 density with less stratification in the confluent state, which initiated maturation with tight junction
4 formation. In the present study, we describe a non-invasive methodology to evaluate the extent of
5 maturation of individual RPE cells by measuring cell migration, and retrospectively analyzing the
6 time- and location-dependent profiles of RPE cells toward the maturation at the central and
7 peripheral regions of the culture vessel.

8 **MATERIALS AND METHODS**

9 **Culture conditions of human RPE cells** Human RPE cells (Lot no. 0F3292; Lonza,
10 Walkersville, MD, USA) were maintained according to the supplier's instructions. The RPE cells
11 were seeded at 1.0×10^4 cells/cm² and expanded in 25-cm² T-flasks (Nunc, Roskilde, Denmark)
12 using RPE growth medium (Cat. No. 00195409; Lonza) at 37 °C in a humidified 5% CO₂ incubator.
13 Medium was changed every 2 days. After reaching 80% confluency, cells were detached by
14 enzymatic treatment with a 0.1% trypsin/0.02% EDTA solution (Sigma-Aldrich, St. Louis, MO,
15 USA). For time-lapse observation, viable cells were seeded at 5.0×10^4 cells/cm² on a
16 laminin-coated surface in 48-well plates (culture area in each vessel was 0.95 cm²; Corning Costar,
17 Cambridge, MA, USA). The laminin-coated surface was prepared according to the supplier's
18 instruction under aseptic conditions. In brief, 10 µg/ml solution (laminin-1; Sigma-Aldrich) in PBS
19 was added to the culture vessel (surface coverage: 2 µg/cm²) and incubated for 2 h at 37°C,
20 followed by washing with PBS prior to cell seeding.

For inhibition of cell migration, RPE cells were exposed to medium with 50 $\mu\text{g/ml}$ Rac1 inhibitor NSC23766 (Calbiochem, Merck, Darmstadt, Germany) for 12 h. The Rac1 inhibitor was added to each well at a concentration of 50 $\mu\text{g/ml}$ at day 5, and was removed after 12 h.

Measurements of cell number and migration rate The protocol to determine time profiles

of cell numbers and migration rate is shown in Fig. 1. The images of each measurement region were Fig. 1

determined from triplicate samples in 48-well plates (Corning Costar). Time-lapse image capture was performed to investigate cell behaviors using an image analyzer with a 10 $\times$ objective lens (IN Cell Analyzer 2000; GE Healthcare, Chalfont St. Giles Buckinghamshire, UK). Original images (1.5 mm $\times$ 1.5 mm) at 16 bits in gray scale and 1.83 pixels/ μm^2 in resolution were captured every 20 min for 6 h at 81 positions on the entire surface. For determination of number and migration of cells at central and peripheral regions, the region of interest (ROI; 300 μm $\times$ 300 μm) was defined in locations at the central and peripheral regions in the culture vessel. The peripheral region was set as the distance of 4.5 mm from the center position of the culture vessel as shown in Fig. 1. The data of cell density, X , and migration rate, V , were obtained from the cells within the central and peripheral regions. The cell densities of central, X_C , and peripheral, X_P , regions were calculated, and the ratio of cell densities at central regions versus those at the peripheral regions, X_C/X_P , were defined as the heterogeneity index for the cell population. For quantification of cell migration, the positional centroids (x_i , y_i) of each cell in the ROI were determined using LabVIEW software (National Instruments, Austin, TX, USA), as previously described (15). The migration rate, V , was defined as an average value for 6 h of migration rates calculated from cell displacements measured every 20 min. The average migration rate, V_M , was calculated from migration rates, V , of 300–1100 cells

1 within triplicate ROIs.

2 **Determination of tight junction formation** For determination of tight junction formation, *in*
3 *situ* analysis for ZO-1 was performed using captured images at the same positions where migration
4 rate was analyzed. A previous report described staining of ZO-1 and the nucleus (9). Briefly, cells
5 were washed twice with PBS and fixed with 4% paraformaldehyde for 15 min at 4 °C. The fixed
6 cells were permeabilized by incubation for 5 min in 0.2% Triton X-100. After washing with PBS,
7 nonspecific binding sites were blocked by treatment with Block Ace (Dainippon Sumitomo Pharma,
8 Osaka) for 1 h. The cells were then treated overnight with anti-rabbit ZO-1 antibody (Abcam,
9 Cambridge, MA, UK) at 4 °C. After washing with Tris-buffered saline (TBS, DAKO, Glostrup,
10 Denmark), cells were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Life
11 Technologies Corporation, Carlsbad, CA, USA) for 1 h. After washing with TBS, the nuclei were
12 stained with 4',6-diamidino-2-phenylindole (DAPI, Life Technologies Corporation) for 20 min at
13 room temperature. The images of cells stained for ZO-1 and nuclei were captured using an IN Cell
14 Analyzer 2000 with 10× objective lens. The fluorescence intensities for ZO-1 and DAPI were
15 obtained by excitation at wavelengths of 488 and 358 nm, respectively.

16 For determination of tight junction formation, individual RPE cells were categorized as either
17 ZO-1-positive cells with a perfectly distinct line of ZO-1 surrounding the cell, or as ZO-1-negative
18 cells with an imperfectly distinct line of ZO-1 surrounding the cell. The frequencies of
19 ZO-1-positive cells and ZO-1-negative cells versus migration rates of the cells, F_X , were estimated
20 in a given range of migration rates.

21 **Statistical Analysis** All experiments were performed at least three times, and the data were

expressed as means with standard deviations. Comparisons between the groups were determined by Student's *t*-test (two groups) or one-way Analysis of Variance (ANOVA) and Tukey-Kramer post-hoc test (more than two groups). *P* values < 0.05 were considered statistical significance.

RESULTS

Change in cell density of RPE cells during maturation Time profiles of cell densities at the central and peripheral regions in the culture vessel were obtained for confluent RPE cells at 21 days after seeding. Fig. 2A shows the ratio of cell density at the central region versus the peripheral region in the same culture vessel. As shown in Fig. 2B, cell densities were the same in the central and peripheral regions at day 1 of culture time, t , ($X = 1.2 \times 10^5$ cells/cm² and $X = 1.1 \times 10^5$ cells/cm², respectively). As cell culture proceeded, the cell density at the central region increased rapidly to $X = 3.4 \times 10^5$ cells/cm² at $t = 5$ d. At $t = 21$ d, cell density of the central region was $X = 3.6 \times 10^5$ cells/cm². At the peripheral region of the culture vessel, cell density gradually increased after a lag period, compared with that at the central region. At $t = 21$ d, cell density at the peripheral region was $X = 3.3 \times 10^5$ cells/cm², which was similar to that at the central region. As shown in Fig. 2A, the heterogeneity index X_C/X_P , the ratio of cell density at the central region versus peripheral region, was $R = 1.1$ at $t = 1$ d, showing the local homogeneity of cellular distribution. This heterogeneity index increased until $t = 3$ d, exhibiting development of local heterogeneity, then started to gradually decrease to $R = 1.1$ at $t = 21$ d.

To characterize the change in maturation of RPE cells, fluorescent staining of ZO-1 and nuclei was performed on days 1, 3, 5, and 21. Fig. 3 shows the time-course of the frequency of

Fig. 2

Fig. 3

ZO-1-positive cells, F_Z , at the central and peripheral regions. At $t = 1$ d, all cells in both regions were negative for ZO-1 expression. As cell culture proceeded, the frequency of ZO-1-positive cells at the central region rapidly increased to $F_Z = 0.75$ at $t = 5$ d. At $t = 21$ d, the frequency of ZO-1-positive cells increased slowly, reaching $F_Z = 0.82$. The frequency of ZO-1-positive cells at the peripheral region began to increase over time. At $t = 5$ d, the frequency at the peripheral region was 63% of the central region. At $t = 21$ d, the frequency of ZO-1-positive cells at the peripheral region increased to 92% of the central region.

Correlation between migration and maturation of individual RPE cells A time-lapse analysis was conducted for RPE cells at the central and peripheral regions (Movie S1). At $t = 1$ d, almost all cells in both regions showed an elongated shape after seeding, which reached confluence through active migration and division. With elapsed culture time, cells at the central region formed a cobblestone-like morphology, gradually becoming small and tight. At $t = 5$ d, cells at the central region showed decreased migration, and cell migration and morphology were maintained until $t = 21$ d. Cells at the peripheral region migrated actively and exhibited large and elongated shapes until $t = 5$ d, compared with those at the central region. As the culture proceeded, cell migration at the peripheral region gradually decreased. At $t = 21$ d, the cell morphology and migration at the peripheral region showed a similar pattern as the central region.

To clarify the correlation between migration and tight junction formation of RPE cells at the central and peripheral regions, the frequencies of ZO-1-positive and ZO-1-negative cells versus migration rates were evaluated. As shown in Fig. 4a, the average migration rate at the central region was $V_M = 15 \pm 4.9 \mu\text{m/h}$ at $t = 1$ d. The average migration rate at the central region began to

Fig. 4

1 decrease with time, and the values decreased to $V_M = 6.3 \pm 3.0 \mu\text{m/h}$ at $t = 3 \text{ d}$ (Fig. 4b). At $t = 5 \text{ d}$,
2 the average migration rate of the central region cells was $V_M = 3.8 \pm 1.8 \mu\text{m/h}$, which was similar to
3 the average migration rate of the central region at $t = 21 \text{ d}$ (Fig. 4c and Fig. 4d). The average
4 migration rate at the peripheral region began to decrease over time, compared with that at the
5 central region. At $t = 21 \text{ d}$, the average migration rate of the peripheral region was $V_M = 3.7 \pm 1.5$
6 $\mu\text{m/h}$, which was the same as the central region (Fig. 4d and Fig. 4h). The migration rates of
7 individual cells in the central and peripheral regions at $t = 1 \text{ d}$ showed a broad distribution in the
8 range of $V = 6.0 \mu\text{m/h}$ to $V = 34 \mu\text{m/h}$ (Fig. 4a and Fig. 4e). The distribution of migration rates at
9 the central region had a narrow range compared with those at the peripheral region (Fig. 4c and Fig.
10 4g). At $t = 21 \text{ d}$, the migration rates at both regions converged to a range of $V = 0\text{--}10 \mu\text{m/h}$ (Fig. 4d
11 and Fig. 4h).

12 At both regions until $t = 21 \text{ d}$, migration rates of ZO-1-positive cells were distributed in the
13 range of $V = 0\text{--}5.0 \mu\text{m/h}$ (Fig. 4A–4D), and the frequency of ZO-1-positive cells in all measured
14 cells showed a 90% agreement with the frequency of cells having migration rates less than 5.0
15 $\mu\text{m/h}$.

16 **Effect of migration on the maturation of RPE cells** The effect of migration on the
17 maturation of RPE cells was studied when exposed to medium with and without $50 \mu\text{g/ml}$ Rac1
18 inhibitor at $t = 5 \text{ d}$. The frequencies of ZO-1-positive cells, F_Z , and cells having migration rates less
19 than $V = 5.0 \mu\text{m/h}$, F_V , were determined by data in cultures with different lots of RPE cells (Fig. 4
20 and Fig. S1). When not exposed to Rac1 inhibitor, the frequencies of ZO-1-positive cells at the
21 central and peripheral regions were significantly different at $F_Z = 0.75$ and $F_Z = 0.47$, respectively

(Fig. 5A). In the central region, after exposure of cells to the Rac1 inhibitor, there were no significant differences in the F_Z and F_V values between Rac1 inhibitor-exposed and non-Rac1 inhibitor-exposed cells (Fig. 5A and Fig. 5B). At the peripheral region, however, the F_Z value of Rac1 inhibitor-exposed cells was significantly higher (1.7-fold) than the non-Rac1 inhibitor-exposed cells (Fig. 5A). The F_Z and F_V values of the Rac1 inhibitor-exposed cells at the peripheral region were the same as those at the central region (Fig. 5A and 5B).

DISCUSSION

Development of local heterogeneity during maturation

The formation and maintenance of tight junctions are critical events that influence the maturation process of cultured RPE cells. For acquisition of time- and location-dependent profiles of maturing RPE cells, we immunolocalized ZO-1 in the same regions where cell behavior was observed. The results demonstrated that division and migration of maturing RPE cells were accompanied by formation and maintenance of tight junctions, resulting in local heterogeneity in cell distribution. The distribution of RPE cells showed transitions from homogeneity to heterogeneity in culture until $t = 3$ d (Fig. 2). The frequencies of ZO-1-positive cells in the central and peripheral regions were significantly different at $t = 5$ d, but not at $t = 3$ d (Fig. 3), when they showed the greatest heterogeneity (Fig. 2). Kokkinopoulos et al. (16) reported that the proliferative capabilities of mouse RPE cells were different in the central and peripheral regions of the culture vessel. In the same paper, they also reported that soluble factors in the culture medium were not capable of influencing this difference. These results are consistent with our results obtained in cultures of RPE

cells at the central and peripheral regions in culture vessels. Taken together, these findings support the hypothesis that locational heterogeneity in maturation can be caused by a difference cell–cell contact due to changes in cultured cell behavior.

Cell behavior during the process of epithelial morphogenesis has been studied in Madin-Darby canine kidney cells. Nobes et al. (17) reported that actively migrating cells without cell–cell contacts form lamellipodia and filopodia at their leading edges, resulting in formation of adherens junctions and tight junctions. The initial cell–cell adhesions were created as a consequence of cell division and collisions between cells with active migration (17-19). As maturation progressed, the cultured cells underwent growth arrest, and adherens junctions and tight junctions became stable (20). If cell–cell junctions were unstable, cells lost a collective migration manner as a result of repetitive short contacts between cells that were resolved and reestablished upon further migration (21, 22), and cells that lost the cell–cell contacts moved independently (23). These studies suggested that RPE cell migration is closely related to the entire maturation process. In the present study, the ZO-1-positive cells had displacement of positional centroids through cell shape changes via interaction between intact cells, and these cells were found to have the migration rates less than $V = 5 \mu\text{m/h}$ (Movie S1). In contrast, the ZO-1-negative cells migrated actively and randomly, and these cells was mostly more than $V = 5 \mu\text{m/h}$ (Movie S1). Regardless of local differences in the culture vessel, the frequency of cells having migration rates less than $V = 5 \mu\text{m/h}$ showed 90% agreement with the frequency of ZO-1-positive cells (Fig. 4). There are no cultural differences between RPE cells with different lot number (Fig. 4 and Fig. S1). The decrease in migration rate at the peripheral region was delayed compared with that at the central region (Movie S1, and Fig. 4A–

D), and the frequency of cells having migration rates less than $V = 5 \mu\text{m/h}$ in the central region was 1.4-fold higher than the peripheral region at $t = 5 \text{ d}$ (Fig. 5B). However, the cell density in the central region was similar to the peripheral region at the same time (Fig. 2). Our results therefore suggest that a major reason for the difference in maturation may be different migratory responses of RPE cells associated with local differences in tight junction formation.

Within the Rho family of small GTPases, Rac1 plays a pivotal role in regulation of migration and cytoskeleton (24). It is well known that high Rac1 activity generates cell elongation and facilitates initial cell-cell contacts (18, 25). In the maintenance of tight junctions, the assembly of macromolecular components at tight junctions appears to be coordinately regulated by different GTPases, with the localization of ZO-1 affected by Rho and Cdc42 activation, but not Rac1 (26). These results support the possibility that inhibition of Rac1 activity is not directly involved in the maintenance of tight junctions in late phase. Rac1 signaling pathways are regulated by selective interactions of guanine nucleotide exchange factor and GTPase activating protein (27, 28). Gao et al. (29) reported that NSC23766 targeting of Rac1 activation by GEF involved a Rac-specific small molecule inhibitor. Thus, we used NSC23766 to investigate the effect of migration on maturation of RPE cells in culture, and the RPE cells were exposed to medium with Rac1 inhibitor at $t = 5 \text{ d}$. The maturation state in the central region was not affected by exposure to Rac1 inhibitor. In contrast, the frequencies of ZO-1-positive cells as well as cells having migration rates less than $V = 5 \mu\text{m/h}$ when exposed to Rac1 inhibitor significantly increased, becoming similar to that at the central region (Fig. 5A and Fig. 5B). Our results suggested that the local difference in maturation state was caused by heterogeneous distribution of cells exhibiting different migration rates. Cessation of cell migration

1 supposedly facilitated stable cell–cell contacts by inhibiting migration, leading to the formation of
2 tight junctions. Together, these findings strongly suggest that cell migration is an important factor
3 affecting the maturation state of RPE cells in culture.

4 **Measurement of cell migration** Time-lapse imaging is a photographic technique that can
5 obtain quantitative data based on cellular properties, such as changes in cell morphology, adhesion,
6 migration, and division, to provide useful information on cellular phenotypes without cell loss (15,
7 30-32). Retrospective analysis based on time-lapse imaging has been used to determine cell fate in
8 embryo development (33, 34). Konstantinos et al. (35) reported that cell tracking analyses were
9 indispensable for understanding processes involving cell populations. These studies led to the
10 possibility that retrospective analysis can be used to understand the maturation process of RPE cells.
11 Thus, we measured the cell migration of individual cells at the central and peripheral regions in the
12 culture vessel, and the ZO-1-positive and ZO-1-negative cells were analyzed retrospectively to
13 characterize changes in migration of RPE cells during maturation. The results showed that cell
14 migration correlated with the maturation state of individual RPE cells. The maturation state of
15 immature RPE cells was induced by inhibition of cell migration, suggesting that the migration rate
16 is a useful parameter to evaluate the maturation state of individual RPE cells in culture.

17 RPE cells are usually cultured in the confluent state to maintain the differentiation capability of
18 the cells. Because it is difficult to analyze the large amount of data obtained from cell tracking
19 analyses, there have been few reports using cell tracking analyses to identify phenotypes of cells in
20 high cell density culture (20). However, cell tracking analysis results in detailed data on changes in
21 the positions of individual cells, providing insight into cell behavior in response to environmental

1 stimuli at different regions in the culture vessel. In the present study, migration measurements of
2 single cell suggested that the migratory behavior of RPE cells caused local heterogeneity of
3 maturation state. Our results demonstrated that time- and location-dependent profiles of individual
4 RPE cell migration were necessary for evaluating the extent of maturation state of these cells in
5 culture. Furthermore, the present study demonstrated that measurement of cell migration is a
6 valuable tool in understanding the time- and location-dependent profiles of individual RPE cells in
7 culture, providing information that could be useful for development and design of the RPE
8 maturation process.

9 In conclusion, cultures of RPE cells were used to evaluate the maturation state of confluent
10 cells, and the correlations between cell migration and maturation at the central and peripheral
11 regions in the culture vessel were determined. The number of ZO-1-positive cells increased with
12 decreasing migration rate. The frequency of ZO-1-positive cells at the central region was 1.6-fold
13 higher than the peripheral region at day 5. Exposure of the cells to Rac1 inhibitor facilitated
14 maturation of RPE cells at the peripheral region due to migratory suppression, showing that cell
15 migration is an important factor affecting maturation of RPE cells, and suggesting that RPE cells
16 showed local heterogeneity of maturation due to changes in migratory behavior in culture.

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REFERENCES

1. **Strauss, O.:** The retinal pigment epithelium in visual function, *Physiol. Rev.*, **85**, 845–881 (2005).
2. **Melville, H. Carpiello, M. Hollis, K. Staffaroni, A. and Golestaneh, N.:** Stem cells: a new paradigm for disease modeling and developing therapies for age-related macular degeneration, *J. Transl. Med.*, **11**, 53 (2013).
3. **Cruz, LD. Chen, FK. Ahmado, A. Greenwood, J. and Coffey, P.:** RPE transplantation and its role in retinal disease, *Prog. Retin. Eye. Res.*, **26**, 598–635 (2007).
4. **Tezel, TH. Del Priore, LV. Berger, AS. and Kaplan, HJ.:** Adult retinal pigment epithelial transplantation in exudative age-related macular degeneration, *Am. J. Ophthalmol.*, **143**, 584–595 (2007).
5. **Osakada, F. Ikeda, H. Sasai, Y. and Takahashi, M.:** Stepwise differentiation of pluripotent stem cells into retinal cells, *Nat. Protoc.*, **4**, 811–824 (2009).
6. **Schwartz, SD. Hubschman, JP. Heilwell, G. Cardenas, VF. Pan, CK. Ostrick, RM. Mickunas, E. Gay, R. Klimanskaya, I. and Lanza, R.:** Embryonic stem cell trials for macular degeneration: a preliminary report, *Lancet.*, **379**, 713–720 (2012).
7. **Krohne, TU. Westenskow, PD. Kurihara, T. Friedlander, DF. Lehmann, M. Dorsey, AL. Li, W. Zhu, S. Schultz, A. Wang, J. Siuzdak, G. Ding, S. and Friedlander, M.:** Generation of retinal pigment epithelial cells from small molecules and OCT4 reprogrammed human induced pluripotent stem cells, *Stem. Cells. Transl. Med.*, **1**, 96–109 (2012).
8. **Li, Y. Tsai, YT. Hsu, CW. Erol, D. Yang, J. Wu, WH. Davis, RJ. Egli, D. and Tsang, SH.:**

Long-term safety and efficacy of human-induced pluripotent stem cell (iPS) grafts in a preclinical model of retinitis pigmentosa, *Mol. Med.*, **18**, 1312–1319 (2012).

9. **Kim, MH. Sonoi, R. Yamada, K. Inamori, M. and Kino-oka, M.:** Analysis of locality of early-stage maturation in confluent state of human retinal pigment epithelial cells, *J. Biosci. Bioeng.*, **113**, 778–781 (2012).

10. **Treharne, AJ. Grossel, MC. Lotery, AJ. and Thomson, HA.:** The chemistry of retinal transplantation: the influence of polymer scaffold properties on retinal cell adhesion and control, *Br. J. Ophthalmol.*, **95**, 768–773 (2011).

11. **Maminishkis, A. Chen, S. Jalickee, S. Banzon, T. Shi, G. Wang, FE. Ehalt, T. Hammer, JA. and Miller, SS.:** Confluent monolayers of cultured human fetal retinal pigment epithelium exhibit morphology and physiology of native tissue, *Invest. Ophthalmol. Vis. Sci.*, **47**, 3612–3624 (2006).

12. **Dunn, KC. Aotaki-Keen, AE. Putkey, FR. and Hjelmeland, LM.:** ARPE-19, a human retinal pigment epithelial cell line with differentiated properties, *Exp. Eye. Res.*, **62**, 155–169 (1996).

13. **Geisen, P. McColm, JR. King, BM. and Hartnett, ME.:** Characterization of barrier properties and inducible VEGF expression of several types of retinal pigment epithelium in medium-term culture, *Curr. Eye. Res.*, **31**, 739–748 (2006).

14. **Gamm, DM. Melvan, JN. Shearer, RL. Pinilla, I. Sabat, G. Svendsen, CN. and Wright, LS.:** A novel serum-free method for culturing human prenatal retinal pigment epithelial cells, *Invest. Ophthalmol. Vis. Sci.*, **49**, 788–799 (2008).

15. **Kino-oka, M. Agatahama, Y. Hata, N. and Taya, M.:** Evaluation of growth potential of

human epithelial cells by motion analysis of pairwise rotation under glucose-limited condition, Biochem. Eng. J., **19**, 109–117 (2004).

16. **Kokkinopoulos, I. Shahabi, G. Colman, A. and Jeffery, G.:** Mature peripheral RPE cells have an intrinsic capacity to proliferate; a potential regulatory mechanism for age-related cell loss, PLoS. One., **6**, e18921 (2011).

17. **Nobes, CD. and Hall, A.:** Rho GTPases control polarity, protrusion, and adhesion during cell movement, J. Cell. Biol., **144**, 1235–1244 (1999).

18. **Yamada, S. and Nelson, WJ.:** Localized zones of Rho and Rac activities drive initiation and expansion of epithelial cell–cell adhesion, J. Cell. Biol., **178**, 517–527 (2007).

19. **Miyoshi, J. and Takai, Y.:** Molecular perspective on tight-junction assembly and epithelial polarity, Adv. Drug. Deliv. Rev., **57**, 815–855 (2005).

20. **Puliafito, A. Hufnagel, L. Neveu, P. Streichan, S. Sigal, A. Fygenon, DK. and Shraiman, BI.:** Collective and single cell behavior in epithelial contact inhibition, Proc. Natl. Acad. Sci. USA., **109**, 739–744 (2012).

21. **Tamiya, S. Liu, L. and Kaplan, HJ.:** Epithelial-mesenchymal transition and proliferation of retinal pigment epithelial cells initiated upon loss of cell–cell contact, Invest. Ophthalmol. Vis. Sci., **51**, 2755–2763 (2010).

22. **Omelchenko, T. and Hall, A.:** Myosin-IXA regulates collective epithelial cell migration by targeting RhoGAP activity to cell–cell junctions, Curr. Biol., **22**, 278–288 (2012).

23. **Hegerfeldt, Y. Tusch, M. Brocker, EB. and Friedl, P.:** Collective cell movement in primary melanoma explants: plasticity of cell–cell interaction, beta1-integrin function, and migration

strategies, *Cancer. Res.*, **62**, 2125–2130 (2002).

24. **McCormack, J. Welsh, NJ. and Braga, Vania MM.:** Cycling around cell–cell adhesion with Rho GTPase regulators, *J. Cell. Sci.*, **126**, 379–391 (2013).

25. **Braga, Vania MM. and Yap, AS.:** The challenges of abundance: epithelial junctions and small GTPase signaling, *Curr. Opin. Cell. Biol.*, **17**, 466–474 (2005).

26. **Bruewer, M. Hopkins, AM. Hobert, ME. Nusrat, A. and Madara, JL.:** RhoA, Rac1, and Cdc42 exert distinct effects on epithelial barrier via selective structural and biochemical modulation of junctional proteins and F-actin, *Am. J. Physiol. Cell. Physiol.*, **287**, C327–335 (2004).

27. **Zheng, Y.:** Dbl family guanine nucleotide exchange factors, *Trends. Biochem. Sci.*, **12**, 724–732 (2001).

28. **Burridge, K. and Wennerberg, K.:** Rho and Rac take center stage, *Cell.*, **116**, 167–179 (2004).

29. **Gao, Y. Dickerson, JB. Guo, F. Zheng, J. and Zheng, Y.:** Rational design and characterization of a Rac GTPase-specific small molecule inhibitor. *Proc. Natl. Acad. Sci. USA.*, **101**, 7618–7623 (2004).

30. **Loerke, D. Duc, QL. Blonk, I. Kerstens, A. Spanjaard, E. Machacek, M. Danuser, G. and Rooij, JD.:** Quantitative imaging of epithelial cell scattering identifies specific inhibitors of cell motility and cell–cell dissociation, *Sci. Signal.*, **5**, rs5 (2012).

31. **Verstraeten, TC. Buzney, SM. Macdonald, SG. and Neufeld, AH.:** Retinal pigment epithelium wound closure in vitro. Pharmacologic inhibition, *Invest. Ophthalmol. Vis. Sci.*, **31**,

- 1 481–488 (1990).
- 2 32. **Umegaki, R. Kino-oka, M. and Taya, M.:** Assessment of cell detachment and growth potential
3 of human keratinocyte based on observed changes in individual cell area during trypsinization,
4 *Biochem. Eng. J.*, **17**, 49–55 (2004).
- 5 33. **Herrero, J. Tejera, A. Albert, C. Vidal, C. de Los Santos, MJ. and Meseguer M.:** A time to
6 look back: analysis of morphokinetic characteristics of human embryo development, *Fertil.*
7 *Steril.*, **100**, 1602–1609 (2013).
- 8 34. **Kirkegaard, K. Agerholm, IE. and Ingerslev, HJ.:** Time-lapse monitoring as a tool for
9 clinical embryo assessment, *Hum. Reprod.*, **27**, 1277–1285 (2012).
- 10 35. **Kokkaliaris, KD. Loeffler, D. and Schroeder, T.:** Advances in tracking hematopoiesis at the
11 single-cell level, *Curr. Opin. Hematol.*, **19**, 243–249 (2012).

1 **Figure legends**

2 FIG. 1. Imaging procedure for determining migration rate and maturation state of confluent RPE
3 cells.

4 FIG. 2. Time-course of cell density (A) and heterogeneity index (B) in the central and peripheral
5 regions of culture at days 1, 3, 5, and 21. Open and closed circles show cell densities at the
6 central and peripheral regions in the culture vessel, respectively. The open triangles show
7 ratio of cell density at the central region versus the peripheral region. The data were
8 obtained from three independent experiments ($n = 3$).

9 FIG. 3. Frequency of ZO-1-positive cells at the central and peripheral regions in the culture vessel
10 on days 1, 3, 5, and 21. Open and closed circles show cell densities at the central and
11 peripheral regions, respectively ($n = 3$). Statistical significance was according to Student's
12 t -test ($*p < 0.05$).

13 FIG. 4. Frequencies of ZO-1-positive and ZO-1-negative cells versus migration rate of RPE cells at
14 the central (a–d) and peripheral (e–h) regions in culture at $t = 1$ d (A), $t = 3$ d (B), $t = 5$ d
15 (C), and $t = 21$ d (D). Closed and open bars show frequencies of ZO-1-positive and
16 ZO-1-negative cells, respectively. The average migration rate and standard deviation were
17 calculated from the data obtained from 300–1100 cells in triplicate culture vessel.

18 FIG. 5. Frequencies of ZO-1-positive cells (A) and cells having migration rates of $V \leq 5$ $\mu\text{m}/\text{h}$ (B) in
19 culture conditions when exposed to medium with (+) and without (-) Rac1 inhibitor at day

5. Closed and open bars show frequencies of ZO-1-positive cells of Rac1 inhibitor-exposed and non-Rac1 inhibitor-exposed cells, respectively ($n = 3$). Statistical significance was according to one-way Analysis of Variance (ANOVA) and Tukey-Kramer post-hoc test ($*p < 0.05$).

Procedure of data analysis

Multi-position capturing of ZO-1 and bright field images

Multi-position image tiling

Decision of the region of interest (ROI)

Measurement of cell number and migration rate

Judgment of ZO-1-positive cells and ZO-1-negative cells versus measured RPE cells

5. Figure 2

5. Figure 3

5. Figure 4

5. Figure 5