Journal of Bioscience and Bioengineering
VOL. 121 No. 2, 220–226, 2016

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

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Received 12 February 2015; accepted 29 May 2015
Available online 28 June 2015

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.

[**Key words:** Human retinal pigment epithelial cells; Maturation; Collective movement; Local heterogeneity; Confluence]

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 cellular retinaldehyde binding protein (CRALBP) accompanied by Na^+, K^+-ATPase pump, and to expression of microphthalmia-associated transcription factor (MITF) with pigmentation (10–12).

Vugler 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/cm^2 on laminin-coated 48-well plates (culture area in each vessel: 0.95 cm^2; 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/cm^2. 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% CO_2 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

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

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2015.05.019

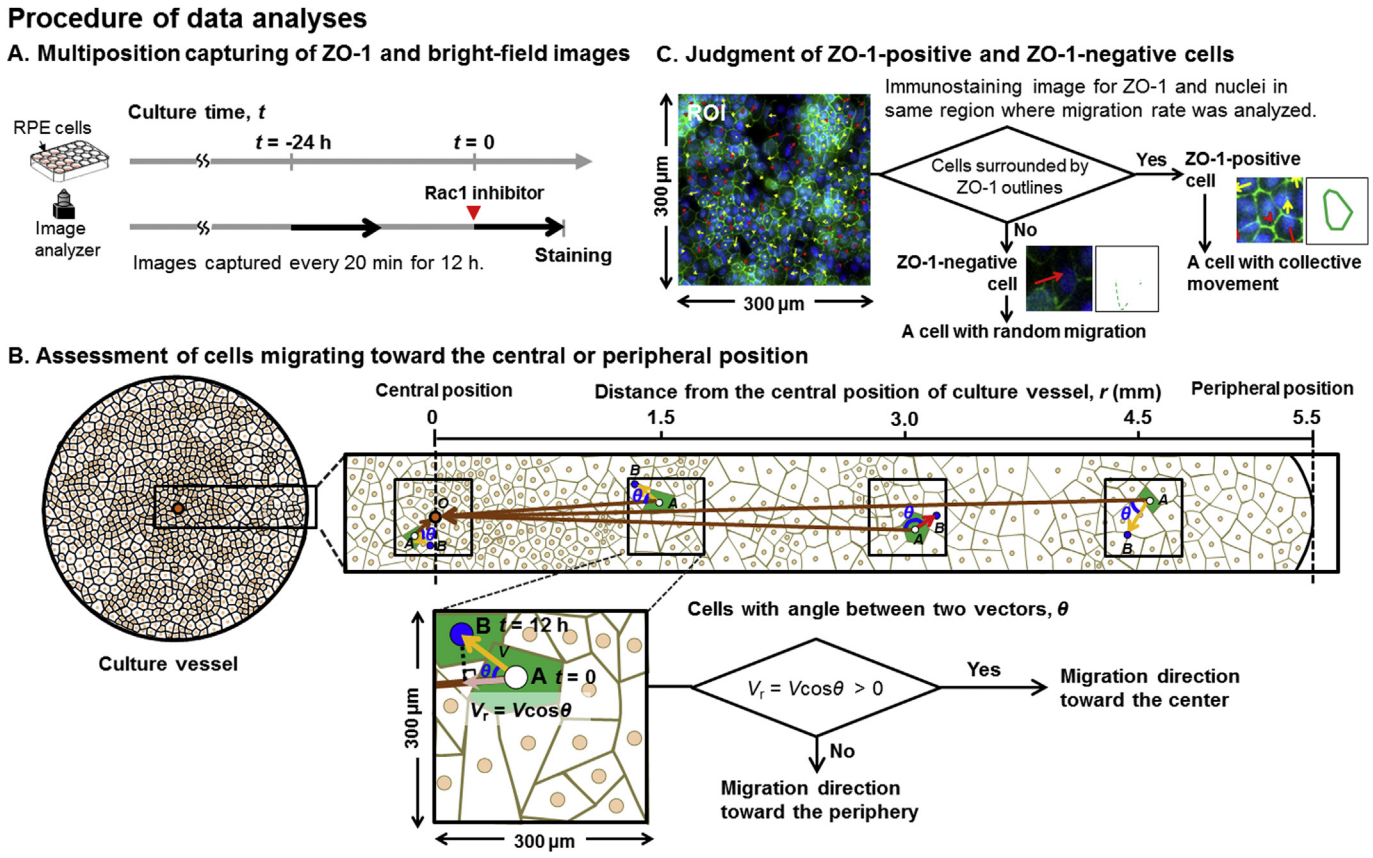

FIG. 1. Procedure of data analyses for the directional migration rate of the cells at each region of interest (ROI) in a culture vessel.

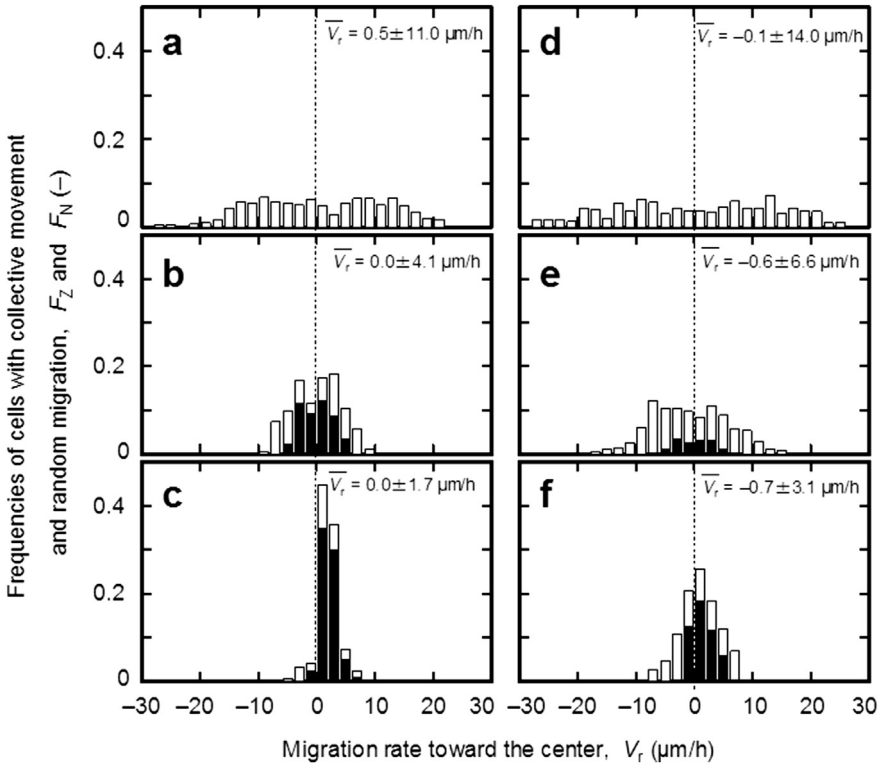

FIG. 2. Frequencies of cells with collective movement and random migration against the migration rate toward the center of individual cells in the vessel at day 1 (a, d), 3 (b, e), and 5 (c, f). Closed and open bars show frequencies of cells with collective movement and random migration, respectively.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