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Locational heterogeneity of maturation by changes in migratory behaviors of human retinal pigment epithelial cells in culture

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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 μm/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 μm/h showed 90% agreement with the frequency of ZO-1-positive cells. To inhibit cell migration, RPE cells were exposed to medium containing 50 μg/ml Rac1 inhibitor at day 5. Frequencies of ZO-1-positive cells and cells having migration rates less than 5.0 μm/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.

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

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

MATERIALS AND METHODS

Culture conditions of human RPE cells Human RPE cells (Lot no. 0F3292; Lonza, Walkersville, MD, USA) were maintained according to the supplier's instructions. The RPE cells were seeded at 1.0×10^4 cells/cm^2 and expanded in 25-cm^2 T-flasks (Nunc, Roskilde, Denmark) using RPE growth medium (Cat. no. 00195409; Lonza) at 37°C in a humidified 5% CO_2 incubator. Medium was changed every 2 days. After reaching 80% confluency, cells were detached by enzymatic treatment with a 0.1% trypsin/0.02% EDTA solution (Sigma–Aldrich, St. Louis, MO, USA). For time-lapse observation, viable cells were seeded at 5.0×10^4 cells/cm^2 on a laminin-coated surface in 48-well plates (culture area in each vessel was 0.95 cm^2; Corning Costar, Cambridge, MA, USA). The laminin-coated surface was prepared according to the supplier's instruction under aseptic conditions. In brief, 10 μg/ml solution (laminin-1; Sigma–Aldrich) in PBS was added to the culture vessel (surface

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coverage: 2 μg/cm^2) and incubated for 2 h at 37°C, followed by washing with PBS prior to cell seeding.

For inhibition of cell migration, RPE cells were exposed to medium with 50 μg/ml Rac1 inhibitor NSC23766 (Calbiochem, Merck, Darmstadt, Germany) for 12 h. The Rac1 inhibitor was added to each well at a concentration of 50 μg/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 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× objective lens (IN Cell Analyzer 2000; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Original images (1.5 mm × 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 × 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 within triplicate ROIs.

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

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

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/cm^2 and $X = 1.1 \times 10^5$ cells/cm^2, respectively). As cell culture proceeded, the cell density at the central region increased rapidly to $X = 3.4 \times 10^5$ cells/cm^2 at $t = 5$ d. At $t = 21$ d, cell density of the central region was $X = 3.6 \times 10^5$ cells/cm^2. 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/cm^2, which was similar to that at the central

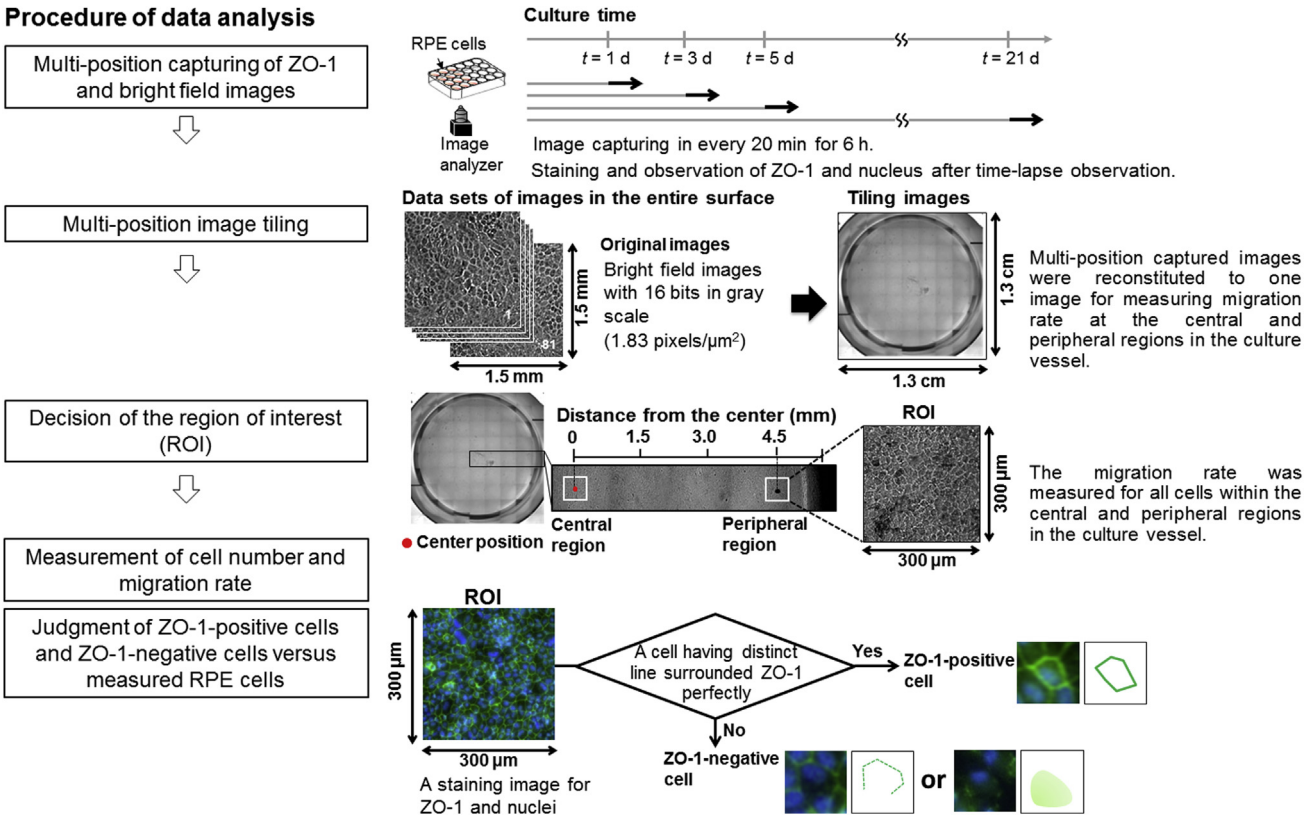

FIG. 1. Imaging procedure for determining migration rate and maturation state of confluent RPE cells.

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