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Phenotypic heterogeneity of human retinal pigment epithelial cells in passaged cell populations

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Human retinal pigment epithelial (RPE) cells at different population doublings (*PD*s) were cultured for 28 days to examine their phenotypic heterogeneity in a confluent state. In an early population (*PD* = 2.8), cells showed a cobblestone-like appearance (type I), which gradually became small and tight, and eventually exhibited dark pigmentation. Some cells showed a dome-like structure (type II), which detached from the culture surface during culture. With increasing *PD*, the cells showed active migration that caused a shift in phenotype from a single layer of large, flattened cells (type III) to a multiple cell layers (stratified) with flattened, irregularly shaped cells (type IV). Immunostaining of specific RPE markers, ZO-1 and Na^+/K^+-ATPase revealed that cells have markedly decreased expressions in a late population (*PD* = 10.1). RPE phenotypes were classified into four types by measuring the nuclear size and local density. The frequencies of type I cells decreased with increasing *PD* value, while the frequencies of type III and IV cells increased along with the decrease in type I. The frequencies of type IV cells at *PD* = 10.1 had increased by 10.3-fold compared with *PD* = 2.8. From these results, the nuclear size and local density were proposed as indicators for understanding phenotypic heterogeneity of RPE cells in the passaged cell population during cell expansion. It is concluded that the population doubling level is an important factor to affect the transition of RPE phenotype and thereby to modulate the quality of cultured cells.

[**Key words:** Human retinal pigment epithelial cells; Population doubling; Heterogeneous cell population; Phenotype; Confluent culture]

The retinal pigment epithelial (RPE) cells, as well as the neural retina, develops from the neuroectoderm and plays a key role in photoreceptor functions (1,2). Several retinal degenerative diseases, such as age-related macular degeneration and retinitis pigmentosa, associated with an impaired RPE function cause the degeneration of the photoreceptor and partial or complete blindness (1). Cultured RPE cells could be a useful source for drug discovery and cell transplantation therapies. In RPE cell cultures, the interactions between cells and the matrix affect cellular morphologies and phenotypes (3–5). Confluent RPE cells form a monolayer with a cobblestone-like appearance and intercellular tight junctions. Disruption of tight junctions in these cells results in loss of cell–cell contacts and concomitant induction of mesenchymal marker expression and cell proliferation (2,6). Over the course of these events, they undergo a significant change in morphology from cobblestone morphology to mesenchymal spindle-like and fusiform features, suggestive of epithelial–mesenchymal transition (EMT) (6–10). EMT is associated with the induction of transcription factors that alter gene expression to promote loss of cell–cell contacts, leading to a shift in cytoskeletal dynamics and a change from epithelial morphology and physiology to the mesenchymal phenotype (6,8). These pathways are activated by various microenvironmental stimuli such as growth factors and cytokines as well as receiving cue from the properties of their cell–cell and cell–matrix interactions. Depending on culture conditions, RPE cells can change their differentiation status, losing cell type-specific features and redifferentiating into epithelial cells. By monitoring the culture process of RPE cells over time, many studies have shown that the cell line, passaging method, passage number, and seeding density have a significant and reproducible effect on RPE cell yield (11–17). There is a need of establishment of methodologies for quantifying phenotypic heterogeneity of RPE cells along the expansion process.

Given the phenotypic variation in the RPE cell culture, it is important to assess the phenotypic heterogeneity using a method that can quantify this variation. Once RPE cells have reached a time point of interest or specific passage number, they are characterized using a variety of assays such as transepithelial resistance measurement, immunofluorescence, gene expression profiling, and methylation analysis (14–22). Most studies of such changes in RPE cell phenotype must rely on time-consuming analysis methods to identify the quality of cells carried out by an experienced technician using extensive assays and tests. Moreover, in some cases of assays require detachment of the cells and are thus disruptive to key characteristics of the cell populations, such as spatial distribution or morphology, preventing collection of this potentially valuable information. However, it is still difficult to apply methods for quantitative measurement of changes in the phenotype of RPE cells in culture. In a previous study, we developed methods to assess the locality of RPE cells at the early stage of maturation by measuring the local density of nuclei in the confluent state, and to make a time-dependent estimation of the extent of maturation during

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confluent culture (14). We have demonstrated that RPE cells at a high nuclear density with less stratification initiate maturation and facilitate formation of tight junctions. In this study, we describe methodology to evaluate phenotypic heterogeneity of RPE cells in passaged cell populations by measuring the nuclear size and local density. Furthermore, cells passaged to various population doublings (*PDs*) were used to understand the mechanism of RPE cell fate determination in the heterogeneous population.

MATERIALS AND METHODS

Preparation of cells with varied population doubling levels Human RPE cells were obtained as frozen cell from Lonza, Walkersville, MD, USA (lot no. 0F3292). The cells in vials were thawed according to the supplier's instruction, and then cultivated in a 25 cm^2 T-flask (Nunclon Delta Flask; Nunc, Roskilde, Denmark). The cultures were conducted using RPE growth medium (cat. no. 00195409; Lonza) at 37°C in a humidified 5% CO_2 incubator. The medium was changed every 2 days. The cells were detached by enzymatic treatment with a 0.1% trypsin/0.02% EDTA solution (Sigma–Aldrich, St. Louis, MO, USA) at about 80% confluence and subsequently applied to passage culture.

In the subsequent subcultures, the cell populations at different *PD* values were prepared in a similar way reported in our previous study (23,24). *PD* was obtained by summation of Δ*PD* with respect to each passage conducted at a seeding density of 1×10^4 cells/cm^2. The total cell number (n_c) was counted under a phase-contrast microscope with a 4× objective lens. At the last day of passage culture, the number of viable cells (n_c) was determined by trypan blue exclusion through direct counting of detached cells. The differential value of *PD* (Δ*PD*) was calculated as follows:

$$\Delta PD = log_2\left(\frac{n_c + \Delta n_c}{n_c}\right) \quad (1)$$

where Δn_c is the differential of number of viable cells in each passage. In this study, viable RPE cells at 24 h after seeding in primary culture, which attached to the surface of the TCPS flask, were defined as a cell population with $PD = 0$.

Incubation of passaged cells in confluent state Cell population at a given *PD* value were seeded at 5×10^4 cells/cm^2 on laminin-coated 48-well plates (culture area in each vessel: 0.95 cm^2; Corning Constar, Cambridge, MA, USA) that were prepared by applying a solution of laminin-1 (Sigma–Aldrich) to the bottom surface at 2 μg/cm^2 according to a method described previously (16). The cells were cultured for 28 days at 37°C in a humidified 5% CO_2 incubator. The medium was changed every 2 days.

Localization of tight junction and Na^+/K^+-ATPase To observe the localization of tight junction and distribution of Na^+/K^+-ATPase in RPE cells, we conducted immunostaining of ZO-1 and Na^+/K^+-ATPase. Cells were washed and then fixed with 4% paraformaldehyde for 15 min at 4°C. After washing with PBS, the cells were permeabilized by incubation for 5 min in 0.2% Triton X-100, and then non-specific binding was blocked by treatment with Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) for 1 h. Cells were then incubated overnight with anti-rabbit ZO-1 antibody (Abcam, Cambridge, MA, UK) and anti-mouse Na^+/K^+-ATPase antibody (Abcam) at 4°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) or Alexa Fluor 594-conjugated anti-rabbit IgG (Life Technologies Corporation) for 1 h. F-actin and cell nuclei were stained with Alexa Fluor 633 phalloidin (Life Technologies Corporation) and 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies Corporation) for 20 min at room temperature. Images were captured using a confocal laser scanning microscope (model FV-1000; Olympus, Tokyo) through a 60× objective lens.

Classification and quantitative analysis of RPE phenotypic variation in confluent state Fig. 1 shows the procedure for data analysis based on the nuclear size and local density. Images of stained cell nuclei and ZO-1 were captured to quantitatively investigate RPE phenotypes using the IN Cell Analyzer 2000 with the 20× objective lens. Original images (1.5 mm × 1.5 mm) at of stained cell nuclei and ZO-1 at 8 bits in gray scale with a 7.3 pixels/μm^2 resolution were captured at the same position. Images were obtained from triplicate samples in 48-well plates. To correlate cellular phenotypes and properties of cell nucleus, we compared frequencies of number of ZO-1-positive cells and nucleus area or local density in the region of interest (ROI; 0.8 mm × 0.8 mm). The density and area of nuclei in the ROI were determined by using the image processing software (Image-Pro Plus version 6.0 software; Media Cybernetics, Silver Spring, MD, USA). The judgment of tight junction formation ZO-1 was conducted by using the captured images at the same positions where nuclei were analyzed. The ZO-1 positive cells were identified as the cells which the distinct line surrounded perfectly, and the local squares with tight junction were estimated to be the region possessing the ZO-1 positive cell. Here, cell nucleus area (A_N) and local nucleus density (X_N) was calculated by total nucleus number in ROIs, and the frequency in number of local squares in a given range of A_N and X_N, F_A and Fx were estimated. And the captured images for double staining randomly obtained more than three images in each well, and whole semiquantitative data were estimated from triplicate samplings. The F_A and Fx values were determined by examining 430–600 cells at $PD = 5.6$ and 10.1 and 500–600 cells at $PD = 5.6$, respectively.

RESULTS

Characterization of phenotypic heterogeneity of RPE cells To investigate the phenotypic heterogeneity of RPE cells, cells at $PD = 5.6$ and 10.1 were cultured on laminin-coated surface for 28 days. Fig. 2 shows the representative images of different

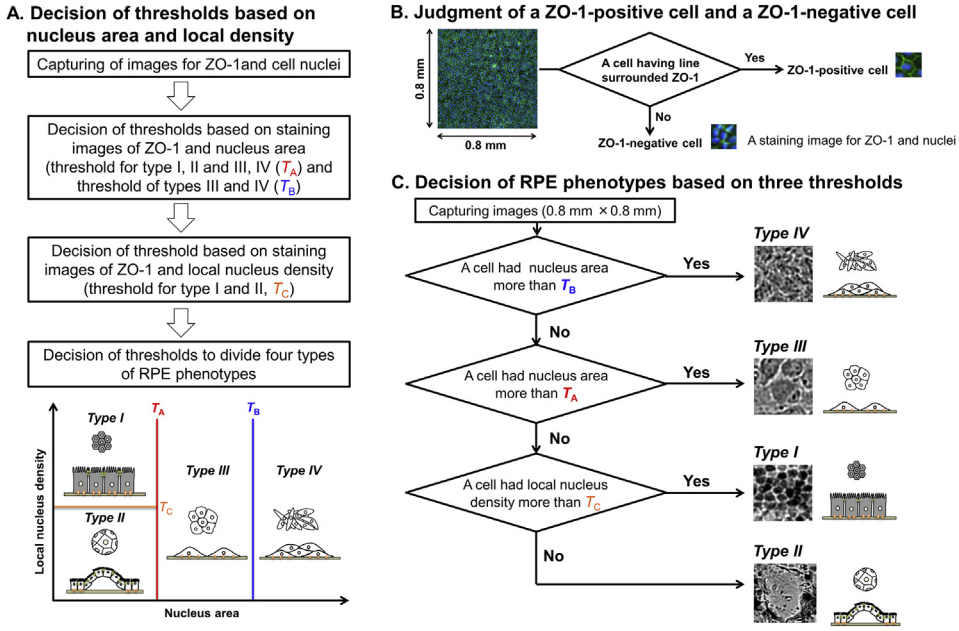

FIG. 1. Procedure for data analyses to determine each phenotype of RPE cells based on nuclear size and local density in the heterogeneous cell population. (A) Whole procedure of data analysis, (B) judgement of a ZO-1-positive cell and a ZO-1-negative cell based on staining images of ZO-1 and nuclei, and (C) decision of thresholds to divide four types of RPE phenotypes. The phenotype of RPE cells was divided into four phenotypes: cobblestone-shaped cells (type I), dome-shaped cells (type II), a single layer of large, flattened cells (type III), and a multiple cell layers with flattened, irregularly shaped cells (type IV).