〈Regular Article〉

# Functional assessment of retinal pigment epithelium cell transplants with various degrees of pigmentation for age-related macular degeneration

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**ABSTRACT** Age-related macular degeneration (AMD) is the major cause of blindness and reduced vision among adults in Japan, for which only symptomatic therapy using an anti-vascular endothelial growth factor (anti-VEGF) drug is currently available. Recently, transplantation of retinal pigment epithelium (RPE) cells derived from human embryonic or pluripotent stem cells has been extensively applied in clinical practice as a radical therapy for patients with AMD; however, its therapeutic effect remains limited. RPE cells have melanin pigment granules that increase over time; moreover, the degree of pigmentation (dPG) increases with the number of pigment granules. The expression levels of RPE-specific genes and the secretion of cytokines reportedly increase as dPG increases. In this study, we performed functional characterization of human RPE (h-RPE) cells with low and high dPG to determine which might be suitable for transplantation to patients with AMD. Specifically, we isolated h-RPE cells with low and high dPG based on evaluation of lightness parameters, then characterized these cells separately. Our results showed that RPE cells with low dPG exhibited elevated phagocytosis and cell adhesiveness, as well as reduced VEGF secretion, compared with RPE cells with high dPG. Because these traits are considered preferable for transplantation to patients with AMD, RPE cells with low dPG may be more suitable for transplantation. Cellular senescence (indicated by levels of senescence-related proteins) was more advanced in RPE cells with high dPG, suggesting that cellular senescence is an important factor that contributes to the suitability of RPE cells for transplantation to patients with AMD.

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Key words：AMD, RPE, Transplant

## INTRODUCTION

Age-related macular degeneration (AMD) is the major cause of blindness and reduced vision among adults in developed countries. In Japan, the incidence of this disease has increased in recent years, such that it has become an important retinal degenerative disease among the major causes of blindness[1)]. Currently, the gold standard therapy for AMD is vitreous injection of an anti-vascular endothelial growth factor (anti-VEGF) drug;

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however, this only constitutes symptomatic therapy, such that remission and withdrawal are unlikely[2, 3]. Additionally, this therapy is occasionally ineffective. Notably, retinal pigment epithelium (RPE) transplantation has attracted great attention as a radical therapy for patients with AMD. Impaired RPE function is presumed to underlie the pathology of AMD. RPE transplantation is able to replenish impaired RPE with healthy RPE. In recent years, transplantation using RPE cells derived from human embryonic stem cells or from human pluripotent stem cells has been extensively applied in clinical practice. A 1-year follow-up study conducted in 2014, regarding the transplantation of human pluripotent stem cell-based RPE into patients with AMD, found no recurrences of exudative changes in the macula, indicating a generally good quiescent condition[4, 5]. However, visual function of recipient patients remains poor after RPE transplantation[6]. To achieve improved visual function after RPE transplantation, there is a need to define the characteristics of RPE cells that are optimal for transplantation.

There are two methods for subretinal transplantation of RPE cells: cell sheet transplantation and sub-retinal injection of cell suspension[5, 7, 8]. Cell sheets exhibit good cell adhesiveness; however, they are highly invasive because they can be up to 1.3 × 3 mm in size, thus requiring insertion through retinal incisions of 1.8 mm[5]. In contrast, cell suspension transplantation allows sub-retinal administration with 38-gauge needles[7] and is less invasive; these characteristics have led to its use as a mainstream surgical technique. However, low cell adhesiveness is a potential limitation of cell suspension transplantation[8].

A key aspect of cell adhesion involves focal contacts. Vinculin proteins form focal contacts, which are used as indicators of cell adhesion[9]. Cell focal adhesions are composed of proteins such as focal adhesion kinase, paxillin, and zyxin, as well as vinculin; staining for any of these proteins reportedly yields nearly the same number and morphology of cells. Thus, vinculin is considered to be a typical protein in the context of cell adhesiveness, which can be useful to assess this characteristic among cells[10]. RPE cells are able to phagocytose photoreceptor outer segments, which is essential for maintenance of retinal homeostasis. Thus, a reduction in the ability of RPE cells to phagocytose photoreceptor outer segments is considered one of the causes of AMD[11]. Moreover, RPE cells are able to secrete VEGF[5], which contributes to choroidal neovascularization in AMD; increased levels of VEGF in the eye are known to aggravate the macular degeneration observed in patients with AMD[2]. Finally, aging has been strongly associated with a decline in RPE cell function. Large amounts of waste products, generated in connection with the decline in RPE cell function, reportedly accumulate in the macula with age[12, 13].

RPE cells are characterized by the presence of melanin pigment granules that increase over time. The degree of pigmentation (dPG) in RPE cells increases with the extent of pigment granules[14]. In addition, the expression levels of RPE-specific genes and the levels of secreted cytokines reportedly increase with increasing dPG in RPE cells; therefore, dPG is regarded as an indicator of RPE maturity[15].

In this study, we attempted to address the problem whereby transplantation of RPE cells cannot improve the visual acuity of patients with AMD; thus, we presumed that the transplantation of RPE cells with suitable quality would contribute to improved visual function in patients with AMD.We hypothesized that cells with high dPG would exhibit improved functional characteristics (e.g., focal contacts, phagocytic capacity, VEGF secretion) that might be suitable for transplantation. Accordingly, we characterized human RPE (h-RPE)

cells with low and high dPG, in terms of their focal contacts, phagocytic capacity, VEGF secretion; we aimed to establish a dPG level in RPE cells that is functionally suitable for transplantation to patients with AMD.

## MATERIALS AND METHODS

*Culture of h-RPE cells*

h-RPE cells were purchased from Lonza (Basal, Switzerland) and cultured on CELLstart-coated dishes (GIBCO, Grand Island, NY, USA) in pre-confluent medium (F10; Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum) before reaching confluence. After reaching confluence, h-RPE cells were cultured in postconfluent medium (DMEM/F12 (7:3) supplemented with B27 (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine (Sigma-Aldrich), and 10 ng/ml basic fibroblast growth factor (Wako, Osaka, Japan).

*Evaluation of the dPG in h-RPE cells*

The methods used for evaluation of the dPG in h-RPE cells were performed as previously described[15]. The RGB value was measured by assessment of the light transmittances at different wavelengths (blue, 436 nm; green, 546 nm; red, 700 nm) using an absorption spectrometer (Varioskan Flash®; Thermo Fisher Scientific, Waltham, MA, USA). The RGB value was rearranged on the basis of the hue, saturation, and value color system, in accordance with the equation provided in previous reports[16]. The lightness of the hue, saturation, and value color system was used to evaluate the dPG in h-RPE cells; the dPG value ranged from 0 to 100, with the blank set as 100. h-RPE cells were classified into two groups: low dPG ($\geq 65$) and high dPG ($<50$). Low- and high-dPG cells were obtained from a single donor.

*Luminescence assay*

h-RPE cells were seeded at a density of $1.0 \times 10^5$ cells/cm$^2$ on 96-well plates. After they had been incubated at 37℃ under 5% $CO_2$ atmosphere for 24 hr, cells were mixed with 100 μl/well CellTiter-GlO® (Promega, Madison, WI, USA). The plates were incubated at room temperature for 10 min in the dark. Relative luminescence units (RLUs) were measured by using a multimode microplate reader (Varioskan Flash®; Thermo Fisher Scientific).

*Immunofluorescence assay*

The methods used for vinculin immunocytochemistry were previously described[17]. h-RPE cells were seeded at a density of $1.0 \times 10^4$ cells/cm$^2$ on 96-well plates. After they had been incubated at 37℃ under 5% $CO_2$ atmosphere for 2 hr, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) and permeabilized with 0.3% Triton X-100™ (Fisher Biotech, Hampton, NH, USA); they were then blocked with Blocking One® (Nacalai, Kyoto, Japan) for 60 minutes at room temperature. Vinculin was detected with a primary antibody (rabbit, 1:50 dilution; Cat. No. 700062, Thermo Fisher Scientific) overnight at 4℃ . Bound primary antibody was detected with Alexa Fluor 488-labeled goat anti-rabbit IgG secondary antibody (1:500 dilution, Cat. No. A27034, Invitrogen). Vinculin-positive focal contacts in each cell were counted using a fluorescence microscope (Olympus BX-53; Olympus, Tokyo, Japan).

*Phagocytosis assay*

h-RPE cells were seeded at a density of $1.0 \times 10^5$ cells/cm$^2$ on 24-well plates. Fluorescein isothiocyanate-labeled beads were added at a final concentration of $2.0 \times 10^6$ beads/ml. After cells and beads had been incubated at 37℃ under 5% $CO_2$ atmosphere for 48 hr, fluorescence-activated cell sorting (FACS) analysis was performed using The BD FACSCanto ™ Ⅱ flow cytometer (Becton Dickson, Franklin Lakes, NJ, USA) to measure the rate of fluorescent bead phagocytosis. FACS

was performed after cells had been washed with phosphate-buffered saline to remove beads that were bound to the cell surface, but had not been phagocytosed. FACS data were analyzed using FlowJo™ software (FlowJo, Ashland, OR, USA).

*Quantitative reverse transcription polymerase chain reaction*

Total RNA was extracted from h-RPE cells using an RNeasy Plus Mini kit® (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. RNA concentration and quality were assessed with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). RNA was reverse transcribed in a 20-μl reaction containing 11 μl of RNA (1000 ng) in DNase- and RNase-free water (Qiagen), 1 μl of 50 μM Oligo (dT) 20 (Invitrogen), 1 μl of 10 mM dNTP Mix (Invitrogen), 1 μl of RNaseOUT (20 U/μl; Invitrogen), and SuperScript™ Ⅲ Reverse Transcriptase (4 μl of 5× First-Standard Buffer, 1 μl of 0.1 M dithiothreitol, and 1 μl of SuperScript Ⅲ at 200 U/μl; Invitrogen). cDNA synthesis was performed using the following reaction conditions: 5 min at 25℃, 60 min at 50℃, and 15 min at 70℃. Quantitative reverse transcription polymerase chain reaction reactions were performed using Ex Taq® DNA polymerase (Takara Bio, Shiga, Japan) and SYBRTM Green master mix (Applied Biosystems, Foster City, CA, USA). The following thermal cycling conditions were applied: one cycle at 50℃ for 120 sec and 95℃ for 120 sec, followed by 40 cycles at 95℃ for 15 sec and 58℃ for 60 sec. Relative expression levels of *MERTK*, an RPE-specific gene, were determined using the $2^{-(\triangle\triangle Ct)}$ method. The following forward and reverse primer sequences (Fasmac, Kanagawa, Japan) were used to amplify the *MERTK* gene: forward, AGGTTGAAGCAGCCCGAAGA; reverse, TGCTTGGTTCCGAACGTCAG.

*Enzyme-linked immunosorbent assay (ELISA) for measurement of VEGF secretion*

The culture medium from confluent h-RPE cells was collected 24 hr after the medium was changed. Secretion levels were measured using a human VEGF-ELISA kit (eBioscience, San Diego, CA, USA), following the manufacturers' instructions.

*Senescence assay*

h-RPE cells were seeded at a density of $1.0 \times 10^5$ cells/cm$^2$ on 96-well plates. In accordance with the manufacturer's instructions, a human IL-6 ELISA kit (R & D Systems, Minneapolis, MN, USA) and a 96-well Cellular Senescence Assay® (Cell Biolabs, San Diego, CA, USA) were used to measure IL-6 expression and Sa-βG activity, respectively. Absorption of 450 nm light was used to measure the expression of IL-6; fluorescence at 360 nm (excitation) / 465 nm (emission) was used to measure the expression of Sa-βG. Both measurements were performed using a multimode microplate reader (Varioskan Flash®; Thermo Fisher Scientific).

*Statistical Analysis*

Data were analyzed with the BellCurve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan).Values are expressed as the mean ± standard error of the mean, and differences with $P < 0.05$ were considered statistically significant. Phagocytosis, luminescence, vinculin, VEGF secretion, and senescence assays were evaluated using the Mann-Whitney U test. Relative *MERTK* gene expression levels were analyzed by using paired t-tests. *$P < 0.05$; **$P < 0.01$

## RESULTS

*Cell adhesiveness*

To clarify the relationship between dPG and cell adhesion, a luminescence assay was used to assess the number of adherent cells among h-RPE cells

with low or high dPG. Luminescence values were 89,954.8 ± 5,987.8 RLUs in the low dPG group and 44,928.6 ± 6,410.2 RLUs in the high dPG group; luminescence was significantly lower in the high dPG group (n = 5 per group, p = 0.0090; Fig. 1a). Vinculin immunocytochemistry was used to determine the number of vinculin proteins (i.e., typical constituents of focal contacts) in h-RPE cells with low or high dPG, which allowed us to examine the relationship between dPG and cell adhesion. The numbers of vinculin-positive focal contacts in each h-RPE cell were 11.6 ± 2.2 in the low dPG group and 3.7 ± 0.9 in the high dPG group; there were significantly fewer vinculin-positive focal contacts in each cell in the high dPG group (n = 11 per group, p = 0.0036; Fig. 1b). Vinculin-positive focal contacts of a h-RPE cell were observed in fluorescence images (right panel, h-RPE cell with low dPG; left panel, h-RPE cell with high dPG; Fig. 1c).

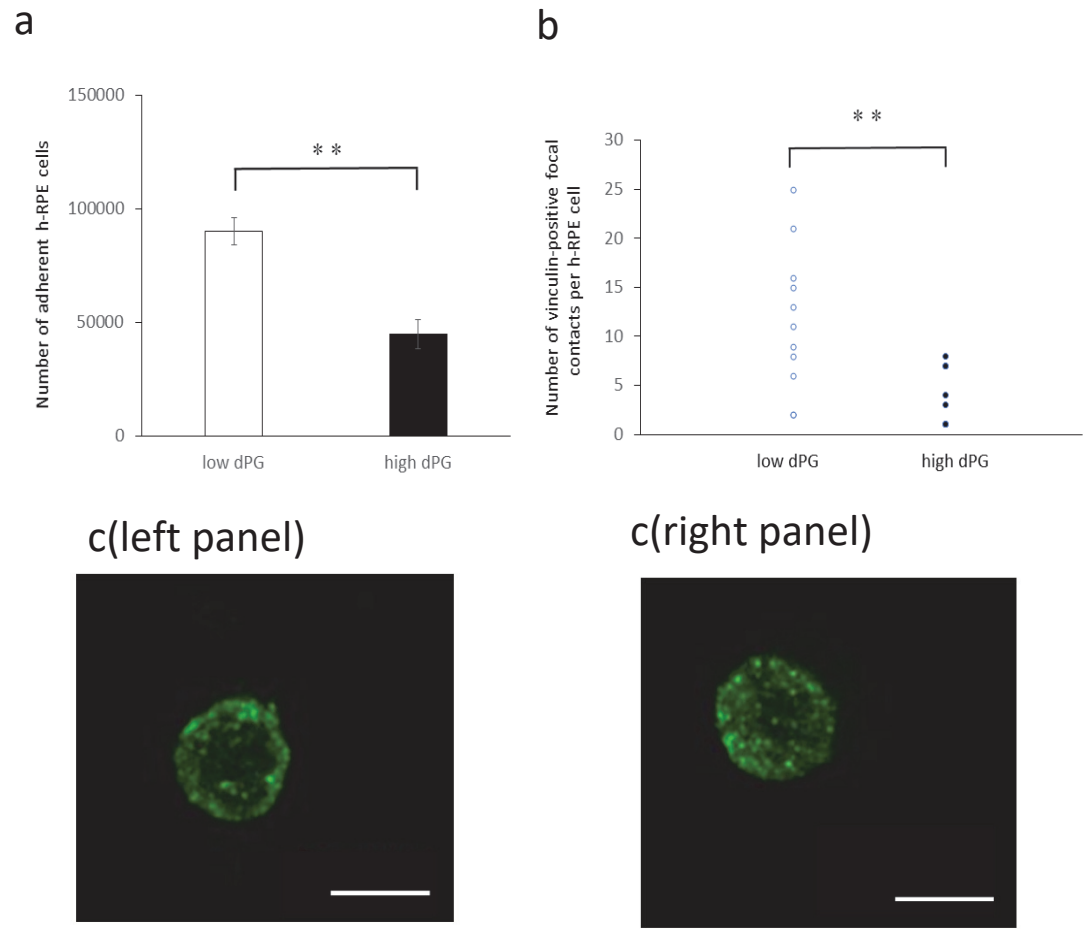


Fig. 1. Evaluation of cell adhesiveness

a. Luminescence assay. The numbers of adherent human retinal pigment epithelium (h-RPE) cells were measured at 24 hr after seeding (n = 5 per group).

b. Immunofluorescence assay. The numbers of vinculin-positive focal contacts per h-RPE cell were counted at 2 hr after seeding (n = 11 per group).

White dots, low degree of pigmentation (dPG) group; black dots, high dPG group

c. Staining of vinculin-positive focal contacts in fluorescence images of h-RPE cells. right panel, h-RPE cell with low degree of pigmentation (dPG); left panel, h-RPE cell with high dPG. Scale bar, 10 $\mu$m.

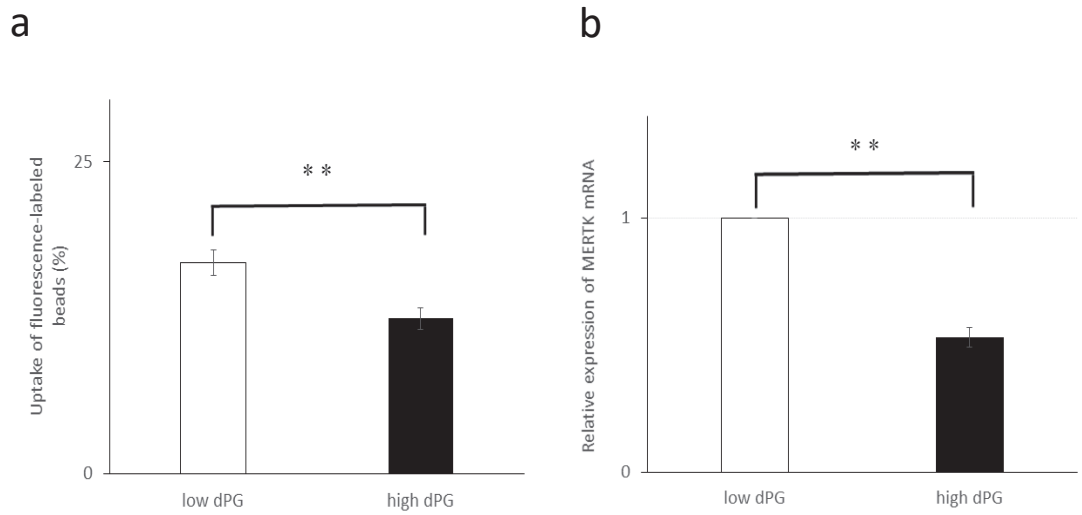


Fig. 2. Evaluation of phagocytic capacity
a. Uptake of fluorescence-labeled beads was measured by fluorescence-activated cell sorting (n = 6 per group).
b. Relative expression of *MERTK* mRNA was evaluated using quantitative reverse transcription polymerase chain reaction (n = 9 per group).
White bar, low degree of pigmentation (dPG) group; black bar, high dPG group

*Phagocytic capacity*

To examine the relationship between dPG and phagocytic capacity, the rates of phagocytosis of fluorescent beads were assessed in h-RPE cells with low or high dPG. Uptake of fluorescence labeled beads was measured by using FACS. The phagocytic rates were 16.9 ± 1.0% in the low dPG group and 12.4 ± 0.8% in the high dPG group; uptake was significantly lower in the high dPG group (n = 6 per group, p = 0.0039; Fig. 2a). Quantitative reverse transcription polymerase chain reactions were used to determine mRNA levels of Mer tyrosine kinase (*MERTK*), a phagocytic marker, in h-RPE cells with low or high dPG, which allowed us to examine the relationship between dPG and phagocytic capability. The expression levels of *MERTK*, a marker of the phagocytic capacity of RPE cells, were evaluated using quantitative reverse transcription polymerase chain reaction assay. The expression level of *MERTK* was lower in the high dPG group than in the low dPG group (n = 9 per group, p<0.001; Fig. 2b).

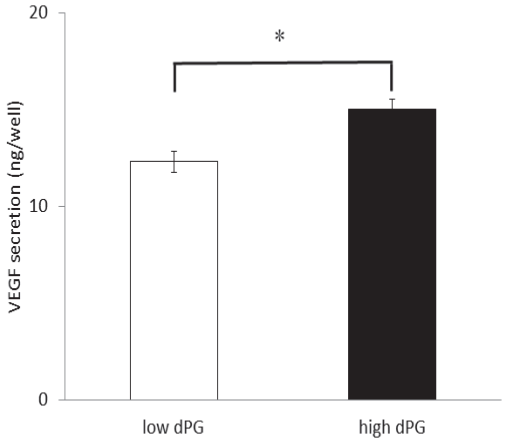


Fig. 3. Assessment of vascular endothelial growth factor (VEGF) secretion
VEGF secretion was evaluated using enzyme-linked immunosorbent assay (n = 6 per group).
White bar, low degree of pigmentation (dPG) group; black bar, high dPG group

*ELISA to measure VEGF secretion*

To examine the relationship between dPG and VEGF secretion, levels of VEGF were determined in h-RPE cells with low or high dPG by using

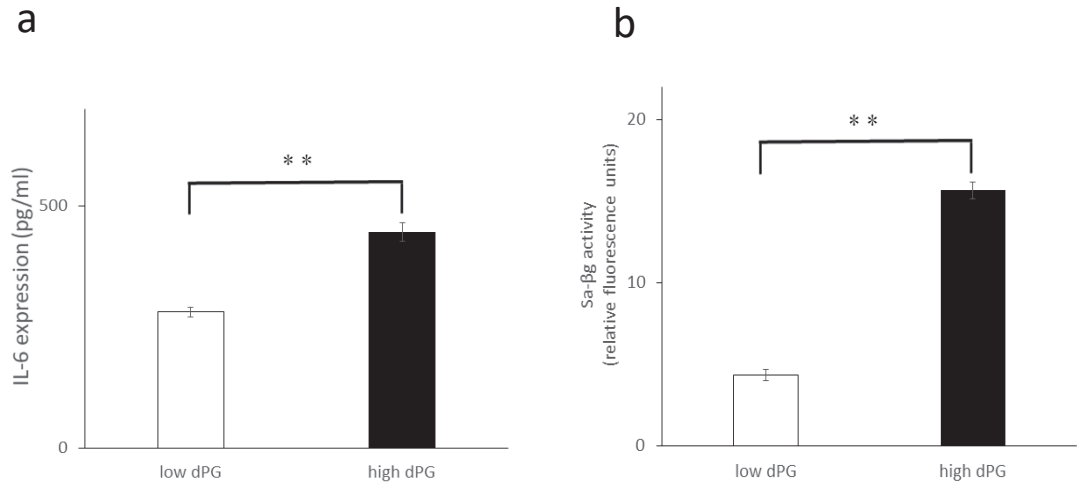


Fig. 4. Evaluation of cell senescence
Cell senescence level was examined by quantitation of interleukin (IL)-6 expression (n = 6 per group (a)) and senescence-associated beta-galactosidase (Sa-$\beta$G) activity (n = 7 per group (b)).
White bar, low degree of pigmentation (dPG) group; black bar, high dPG group

ELISA analysis. Secretion of VEGF was evaluated using ELISA. The levels of VEGF were 12.3 ± 0.6 pg/ml in the low dPG group and 15.0 ± 0.5 pg/ml in the high dPG group; secretion of VEGF was significantly lower in the low dPG group (n = 6 per group, p = 0.016; Fig. 3).

*Cell senescence*

To clarify the relationship between dPG and cell senescence, levels of senescence-associated beta-galactosidase (Sa-$\beta$G) and interleukin (IL)-6 were quantitatively analyzed in h-RPE cells with low or high dPG, by using a senescence assay. The level of cell senescence was examined by quantitation of IL-6 expression and Sa-$\beta$G activity. The expression levels of IL-6 were 281.1 ± 10.7 (pg/ml) in the low dPG group and 446.3 ± 19.4 (pg/ml) in the high dPG group; expression of IL-6 was significantly higher in the high dPG group (n = 6 per group, p = 0.0039; Fig. 4a). The relative fluorescence units of Sa-$\beta$G activity were 4.3 ± 0.3 in the low dPG group and 15.7 ± 0.5 in the high dPG group; Sa-$\beta$G activity was significantly higher in the high dPG group (n = 7 per group, p = 0.002; Fig. 4b).

**DISCUSSION**

This study aimed to determine the dPG in RPE cells that are suitable for transplantation to patients with AMD by separately assessing the functions of RPE cells with low and high dPG. Expression of the *MERTK* gene, a marker of the phagocytic capacity of RPE cells, was lower in RPE cells with high dPG, compared with RPE cells with low dPG. The rate of fluorescent bead phagocytosis and the capacity for cell adhesiveness were also lower in RPE cells with high dPG. Furthermore, secretion of VEGF, a key factor in the onset of AMD pathology, was enhanced in RPE cells with high dPG. These results suggest that the RPE cells with low dPG might be suitable for transplantation to patients with AMD, based on the cell biological data shown here. In addition, RPE cells with high dPG showed higher levels of cellular senescence markers (i.e., Sa-$\beta$G activity and IL-6 expression), compared with RPE cells with low dPG; this finding indicated the advancement of senescence in RPE cells with high dPG, compared with RPE cells with low dPG. Thus, cellular senescence may underlie the reductions of phagocytosis and cell adhesiveness, as well as the

enhancement of VEGF secretion, in RPE cells with high dPG.

Royal College of Surgeons rats, which exhibit abnormal RPE phagocytic capacity, form a normal retinal layer structure before birth; however, outer segments of photoreceptor cells accumulate by approximately 1 month after birth, resulting in photoreceptor cell degeneration[18]. This genetic abnormality in Royal College of Surgeons rats is known to be caused by the aberrant expression of *MERTK*, a tyrosine kinase receptor[19]. Additionally, reduction of the phagocytic capacity of RPE cells due to AMD, along with the accumulation of cell debris in the retina, is presumed to generate oxidative stress that causes chronic inflammation, eventually leading to choroidal neovascularization[20]. Therefore, reduced phagocytic capacity is undesirable for cells that will be transplanted to patients with AMD.

The dPG in RPE cells increases over time; therefore, it is regarded as an indicator of RPE maturity[11]. Furthermore, secretion of cytokines and expression of RPE-specific genes reportedly increase in relation to enhanced dPG in the RPE[5]. Thus, we hypothesized that RPE cells with high dPG would have improved cellular function, compared with RPE cells with low dPG, which would indicate that RPE cells with high dPG might be suitable for transplantation to patients with AMD. However, our results did not support this hypothesis. This surprising finding is valuable, in that it contradicts the existing notion that RPE cells exhibit increased function with increased dPG.

We speculate that cellular senescence is involved in the mechanism that causes reduction of phagocytic capacity and cell adhesiveness in RPE cells with high dPG, combined with the increased secretion of VEGF by these cells. Indeed, aging is presumed to contribute to the reduction of the phagocytic capacity of RPE cells, which leads to the onset of AMD. Moreover, compared with normal eyes, eyes with AMD reportedly show greater accumulation of lipofuscin, the senility pigment[21]; drusen accumulate in the macula as waste products due to the reduced phagocytic capacity of RPE cells[20, 21]. With respect to cell adhesiveness and cellular senescence, matrix metalloproteinase, which increases during aging of RPE cells, has been shown to induce integrin inactivation by processing extracellular matrix, thereby attenuating cell adhesiveness[22]. IL-6 exhibited significantly elevated expression in RPE cells with high dPG; this cytokine is known to enhance VEGF expression[23].

Transplantation of RPE cells has not achieved improved visual acuity in patients with AMD, although it has been successful in reducing disease activity. Thus, we presumed that transplantation of RPE cells with suitable quality could improve visual function in patients with AMD; this study was conducted to identify RPE cells with improved function. Our results suggested that RPE cells with low dPG might serve as high-quality transplants. Therefore, the identification of cells with low dPG and suitable quality, prior to transplantation, shows promise for improving visual function and long-term prognosis in patients with AMD.

An important limitation of this study is that it only involved *in vitro* cell culture experiments; thus, the findings must be confirmed in future animal experiments. Specifically, the effects of phagocytosis, cell adhesiveness, and VEGF must be assessed *in vivo* after subretinal transplantation of RPE cells with high or low dPG to experimental animals. An additional limitation should be noted regarding the phagocytosis assay: FACS was performed after cells had been washed with phosphate-buffered saline to remove beads that were bound to the cell surface, but had not been phagocytosed. Importantly, this procedure did not allow full discrimination of phagocytosed beads from bound beads, which may have influenced the phagocytosis findings.

RPE cells with low dPG showed elevated phagocytosis and cell adhesiveness, as well as reduced secretion of VEGF, compared with RPE cells with high dPG, suggesting that the RPE cells with low dPG might be suitable for transplantation to patients with AMD, based on the cell biological data shown here. Our results also showed that cellular senescence was more advanced in RPE cells with high dPG than in RPE cells with low dPG, suggesting that cellular senescence is involved in the mechanism that causes reduction of phagocytic capacity and cell adhesiveness in RPE cells with high dPG, combined with the increased secretion of VEGF by these cells.

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## CONFLICT OF INTEREST

The authors have no conflicts to disclose.

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